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SOME EFFECTS OF CANCER-PRODUCING AGENTS ON CHROMOSOMES.

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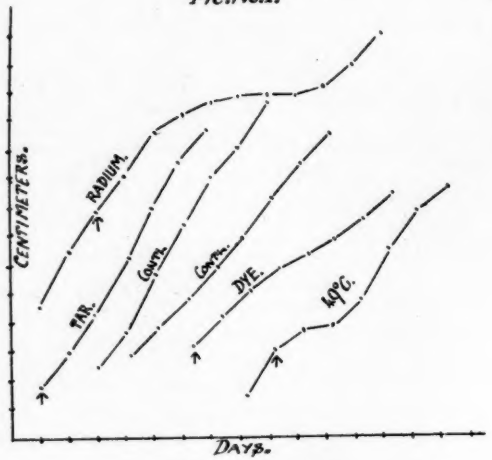
THE mutation hypothesis for cancer postulates that the cancer-cell differs from the normal cell in its content of genes. If such be the case, then the most likely cause of such abnormality is undoubtedly some disturbance during mitosis in the regular distribution of genes to the daughter-cells. It is not possible to see disturbances which affect single genes or even a few genes, since genes are too small to be seen microscopically. If, however, under experimental conditions visible disturbances occur, affecting whole chromosomes, or parts of chromosomes, then it may be assumed that lesser invisible disturbances affecting one or a few genes also occur. There is evidence that this is indeed the case; for instance, X-radiation causes visible chromosomal disturbances, and it also causes invisible ones affecting one or a few genes, as shown by breeding experiments carried out with the radiated sex cells of plants and animals, and with radiated somatic cells in the case of plants and in a few occasions in animals. This being so, it is of interest to ascertain whether agents known to be associated with the origin of cancer are potent to give rise to visible disturbances in mitosis, and more especially to unequal distribution of chromosomes, or parts of chromosomes, to the daughter-cells.

Growing bean roots have therefore been subjected to the following agents: Gamma radiation from radium, heat, a solution of tar, and a solution of gentian violet. The conditions of the gamma radiation were: Exposure to 60 mgm. radium for 1¼ hours; area a square of 4 sq. cm.; screen 3 mm. lead; distance 2 mm. The heated beans were subjected to water at 49° or 50° C. for half a minute. The tar solution was obtained by taking 1 gm. of a cancer-producing tar, dissolving this in 10 c.c. ether and shaking up with 1000 c.c. tap-water. The ether was then evaporated off and the beans grown in this solution for from 1 to 5 days. The gentian violet solution was 0·0025 per cent. and the beans were exposed for 48 hours.

Fig. No. 1.



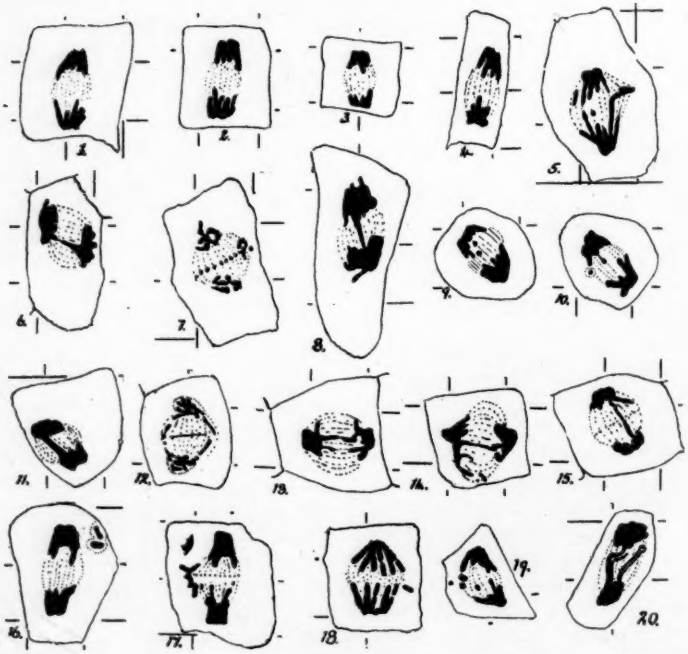


Fig. 2.

The beans were fixed in Bouin's solution and stained with iodine gentian violet, or iron-hæmatoxylin.

These treatments did no more than temporarily decrease the rate of growth of the roots, as shown in Fig. 1.

Attention was confined to the late anaphase stage of mitosis when irregularities in distribution of chromosomes are best seen. Illustrations of these are shown in Fig. 2, the scale being given by the lateral marks, which are 15μ apart.

Nos. 1–4 show four normal cells at this stage. No irregularities were ever seen in normal bean roots. The remaining figures show deviations from the normal.

Nos. 5–7, three anaphases three days after exposure to γ radiation; Nos. 8–11 after exposure to heat; Nos. 12–18 after being grown in tar solution, and Nos. 19 and 20 in gentian violet.

The figures show fragmentation of chromosomes and their delayed migration to the poles of the spindle. These changes occur, as is well known, under many other experimental conditions, such, for instance, as exposure to acids and anæsthetics, high concentration of CO_2, high osmotic pressure, fertilization with foreign sperm, etc.; they are not therefore peculiar to cancer-producing agents. If cancer-producing agents produce cancer by acting upon genes, then tar, when painted on the skin for instance, may differ from many other agents which affect mitosis but do not cause cancer, in being able to penetrate the horny layers and act upon the living basal layer of cells.

It may be that some such property as this determines whether or no a substance is cancer-producing, and not upon some peculiar direct action of them on living cells.

THE INFLUENCE OF ISCHÆMIA ON THE DEVELOPMENT OF TUMOURS.

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PRIMARY malignant disease is comparatively rare in certain organs and tissues. Speaking broadly, it may be said that in many instances, *e. g.* spleen, liver, heart, voluntary muscle, such sites are amongst the most vascular in the body. Tumours are common at the hilum of the lung, while the highly vascular lobes are much less often involved, in spite of the fact that the frequency of metastases in them seems to show that they offer little resistance to the proliferation of malignant cells. In the small intestine, where cancer is rare, the fluid contents and continuous mesentery offer less opportunity for circulatory

interference than is the case in the large intestine, where the mesentery is interrupted, and where cancer is common. Jeanneny (1932) finds that carcinoma of the skin of the face is most frequent in the least vascular areas. Carcinoma of the œsophagus generally involves those parts most subject to pressure from other structures.

The association between scar-tissue and the development of tumours has long been recognized, and has been adduced in support of the chronic irritation theory of cancer. The possibility here exists that the ischæmia may play some part, as fibrotic lesions are nearly always less vascular than the tissue they have replaced. In syphilitic leukoplakia of the tongue, an important precancerous condition, endarteritis is an essential histological part of the lesion. The carcinogenic action of X-rays may conceivably be partly related to the obliterative changes they produce in vessels.

The most striking instance of a lesion associated on the one hand with cancer and on the other with a circulatory defect is that of cirrhosis of the liver. Stewart (1931) has shown that carcinoma is 66 times commoner in cirrhotic than in non-cirrhotic livers, and, further, that the incidence of cirrhosis is significantly higher in liver-cell than in bile-duct cancer. The principal extra-hepatic changes at necropsies on cancer of cirrhosis of liver are those of portal obstruction, and in the liver itself one would expect any effect to operate most markedly on the most vascular tissue, *i. e.* the parenchyma cells.

Further similar considerations, which need not be detailed here, also suggested that tumours are more likely to arise in tissues where the vascular supply is interfered with, and it was decided to attempt to test this hypothesis experimentally by observing the effect of vaso-constrictor drugs on the induction of tumours in mice by a carcinogenic agent. The carcinogenic agent employed was an ether extract of a gas-works tar, previously treated with lime. The vaso-constrictor drugs used were ephedrine sulphate in 2·5 per cent. solution, and adrenalin hydrochloride solution (B.P.) diluted tenfold immediately before use, *i. e.* 0·01 per cent. adrenalin. The earlier experiments were done with ephedrine, as it was thought that the more prolonged action would be of greater importance than the more intense contraction obtained by adrenalin. In the first two experiments the mortality was very high, owing to a series of epidemics, but subsequent isolation of mice in separate glass jars gave a much better survival rate.

The injections were made immediately before applying the tar at weekly intervals. Over a small area of skin between the scapulæ of mice the hair was clipped close, the needle was introduced in the mid-line at the caudal end of the clipped area and passed in the skin to its centre. The object of this manœuvre was to exclude as far as possible effects due to the actual trauma of injection by standardizing its situation. An attempt was made to deliver as much as possible of the injection intra-cutaneously. The amount injected was 0·05 c.c. (= 1·25 mgm. ephedrine and 0·005 mgm. adrenalin). Controls were given a similar injection of 0·05 c.c. of physiological saline, and further controls were tarred without any preliminary injection. The results are shown in Table I. Tumours were recorded as from the week of their first appearance, but warts which regressed later were subsequently excluded in tabulating the results. This phenomenon of regression was rather more frequent in the

TABLE I.

Weeks from first application of tar.	Tar only.			Tar + saline.			Tar + ephedrine sulphate.			Tar + adrenalin hydrochloride.		
	S.	T.	%.	S.	T.	%.	S.	T.	%.	S.	T.	%.
0	125	..	..	150	..	..	150	..	..	51	..	..
5	108	..	..	108	..	..	93	1	1	50	..	..
6	104	..	..	104	..	..	90	5	6	49	..	..
7	103	..	..	103	1	1	88	7	8	49	1	2
8	85	..	..	81	2 (+1)	2	74	5 (+2)	7	49	5	10
9	70	1	1	77	3 (+1)	4	69	6 (+2)	9	49	8	16
10	67	1	1	73	3 (+1)	4	62	9 (+2)	15	48	12	25
11	65	4	6	72	4 (+1)	6	61	12 (+3)	20	48	17	35
12	53	5	9	69	5 (+1)	7	55	14 (+3)	25	47	22 (+1)	47
13	51	9	18	69	12 (+1)	17	55	19 (+3)	35	47	24 (+1)	51
14	46	13 (+1)	28	69	18 (+1)	26	53	24 (+3)	45	47	30 (+1)	64
15	43	17 (+2)	40	68	26 (+1)	38	52	26 (+3)	50	46	33 (+2)	72
16	40	18 (+3)	45	65	36 (+2)	55	51	34 (+4)	67	44	38 (+3)	86
17	38	18 (+4)	47	65	38 (+2)	58	50	38 (+4)	76	43	38 (+4)	88
18	36	23 (+5)	64	65	46 (+2)	71	49	40 (+5)	82	42	38 (+5)	90
19	36	26 (+5)	72	64	48 (+2)	75	48	42 (+6)	88	39	37 (+7)	95
20	32	26 (+7)	81	63	52 (+2)	83	47	41 (+7)	87	37	36 (+9)	97

S. = Total survivors. T. = Survivors with tumours. The figures in parentheses indicate the number of dead tumour-bearing animals up to date. The sum of the two is therefore the total tumours produced in the given period of time. % = The percentage of total survivors having tumours.

ephedrine series, so that if such warts are considered there would be a slightly greater acceleration of tumour induction in this group. For brevity all these experiments have been combined in one table, and this explains the smaller number of adrenalin animals; it should, however, be pointed out that in all three individual experiments the ephedrine-injected mice developed tumours slightly earlier than either of the controls; this seems to indicate that the rather slight difference is not necessarily insignificant. The figures with adrenalin are suggestive, and this substance will be the subject of further investigations. It is scarcely permissible to draw any conclusions from the ephedrine results. The simple injection of saline does not seem to have much effect on the rate of appearance of tar tumours. The behaviour of the non-injected controls is comparable with previous experience of the same tar in the hands of other workers in this laboratory. It will be more convenient to deal with the malignancy of the tumours later.

TABLE II.—*Tar after Ephedrine.*

Weeks.	Survivors.	Tumours.	Percentage.
0	30	..	..
5	26	1	4
6	26	4	15
7	25	5	20
8	25	6	24
9	25	7	28
10	25	11	44
11	25	12	48
12	24	14	58
13	24	18	75
14	24	19 (+1)	79
15	21	18 (+3)	86

During the first of the above experiments, control mice were injected weekly with ephedrine sulphate, in a similar manner to the other injections, but no tar was applied. After 18 weeks there were 30 of these mice left, and it was decided to discontinue the ephedrine injections, and, after an interval of 2 weeks, start applying tar at weekly intervals. The result, which is indicated in Table II, showed an unexpected acceleration of the tumour induction rate. Two explanations seemed possible: (1) That the ephedrine had effected chronic changes in some or all of the epithelial cells; (2) that the subcutaneous fibrosis resulting from the injections was responsible. The first explanation appeared untenable on general grounds, and in view of the fact that the tumours appeared earlier than when the ephedrine injections were continued throughout the period of tar application. The second explanation, therefore, seemed more probable, and an experiment was devised to test it.

In a large group of mice the hair between the scapulæ was clipped short, and, with aseptic precautions, linen thread sutures were introduced immediately beneath the skin. The needle was introduced at the periphery of the clipped area, and carried under the skin to the other side, where it emerged and was

re-introduced. In this way the sutures were taken three or four times in different directions beneath the skin, all the punctures being kept well to the periphery of the area involved. The thread was left in for five weeks, at the

TABLE III.

Weeks from first application of tar.	Mice which had preliminary introduction and removal of sutures.			Control mice. Tar only.		
	S.	T.	%	S.	T.	%
0	68	..	..	50	..	..
4	63	1	2	49	..	..
5	61	2	3	49	..	..
6	61	3	5	49	..	..
7	61	3	5	49	..	..
8	60	8	13	31	..	
9	59	10 (+1)	17	31	1	3
10	58	19 (+1)	33	31	1	3
11	57	29 (+1)	51	31	3	10
12	53	30 (+3)	57	31	4	13
13	53	37 (+3)	70	31	5	16
14	51	41 (+4)	80	31	7	23
15	49	42 (+5)	86	31	12	39
16	47	46 (+5)	98	31	14	45
17	43	43 (+9)	100	31	15	48
18	42	42 (+10)	100	29	19 (+1)	66
19	41	41 (+11)	100	29	22 (+1)	76
20	38	38 (+14)	100	28	22 (+2)	79

TABLE IV.—*Histology of Tumours. During 21st week of experiment, except e (during 16th week.)*

	Malignant.	Doubtful.	Benign.	Percentage of total survivors showing malignancy.
a. Tar only	4	1	22	12·5%
b. Tar + saline	12	12	28	19%
c. Tar + ephedrine	8	4	30	17%
d. Tar + adrenalin	13	3	20	35%
e. Ephedrine alone 18 weeks, then tar alone 15 weeks	2	6	10	10%
f. Tar after sutures	9	6	23	24%

In the interval between the period covered by Tables I and III and the killing of the mice, one further tumour each appeared in Groups *a* and *c*.

Metastases were found in two animals, both belonging to Group *c*. In one case the scapular lymph-gland was involved, in the other lymph-gland and lungs.

end of which time it was removed, and after a further 2½ weeks healing appeared to be complete in most of the mice, histological examination of a few revealing an increase in collagen fibre, with no persistent cellular reaction.

Animals showing any evidence of ulceration or active inflammation were discarded. The remaining 68 mice were subjected to weekly tarring, the tar being applied over a smaller area than that previously occupied by the sutures, in order to exclude the complication of effects due to damage to the epithelium at entrance and exit wounds during suturing.

The results of this experiment are shown in Table III. There is quite a definite acceleration in the appearance of tumours, thus tending to confirm the provisional explanation of the results shown in Table II.

The malignancy of the tumours was decided by histological examination (Table IV). The criterion of malignancy was invasion of the panniculus carnosus. Tumours which appeared to be malignant but did not show infiltration of the muscle have been classified as doubtful, though many of them were probably malignant. The animals were all killed during the 21st week of tar-painting, with the exception of the group in which ephedrine was discontinued before tar applications were started, the latter being killed during the 16th week. To make the figures for the various groups as comparable as possible no account is taken of mice which died during the experiment.

While the figures only warrant tentative conclusions, carcinogenesis appears to have been accelerated by the local action of adrenalin hydrochloride, and by the preliminary fibrosis produced by the introduction of linen thread sutures. The result of the latter procedure is open to a variety of more or less hypothetical explanations, but the devascularization of the part as a consequence of the presence of scar-tissue seems to be an aspect that demands further investigation. The question of active inflammatory changes does not arise, as tarring was not begun till these had subsided. It is also unlikely that direct stimulation of the epithelium was responsible, as the sutures only came into contact with it well outside the area affected, and were no longer present at the time the carcinogenic agent was being applied.

The vascular aspects of experimental tar cancer received attention from Kreyberg (1929) and Guldberg (1931). Kreyberg laid stress on the permanent nature of the hyperæmia produced by tar, and regarded this as the important process. He found that ectases were formed in the course of the vessels supplying the tarred area of skin, and in many of these angiectases were formed thrombi. The latter would suggest that there was a considerable degree of stasis, so that although much blood was histologically found in the vessels, the hyperæmia may well be of a passive nature, and the circulatory efficiency of the part much impaired.

When tar is applied to the skin of the mouse, hyperplasia of the epithelium is found in the course of a few days. This simple hyperplasia reaches its maximum almost at once, and it is not until some weeks later that there is evidence, in the appearance of warts, of neoplastic proliferation. By this time changes have taken place in the deeper tissues, resulting in a fibrous thickening of the corium and subcutis, and it is more than probable that this results in more or less strangulation of some of the vessels. It is suggested that the tumours arise from the stimulating action of tar at the resulting ischæmic points in the skin. Such a conception would explain the fact that tar tumours never arise in the whole of the area painted, but show a focal distribution.

If ischæmia is of importance in inducing the neoplastic change in cells, the reduction in oxygen supply may be the determining factor. This is supported by the conclusion arrived at by Warburg (1930) as a result of his investigations on the metabolism of normal and malignant tissues, *i. e.* that "interference with the respiration in growing cells is, from the standpoint of the physiology of metabolism, the cause of tumours".

SUMMARY.

1. Fibrous scar-tissue was produced in the subcutaneous tissues of mice by the insertion of linen thread sutures, which were subsequently removed, and healing allowed to take place. Care was taken to avoid direct injury to the epithelium. Tar applications induced tumours more rapidly in these mice than in controls, and histological examination showed that the percentage of carcinoma at the 21st week was twice that in the controls.

2. Local injection of vaso-constrictor drugs produced an acceleration in tumour-induction, more marked with adrenalin hydrochloride than with ephedrine sulphate. Carcinoma was increased with adrenalin, but not significantly with ephedrine, as compared with saline-injected and non-injected controls, at the 21st week of tarring.

3. Tumours appeared more rapidly than usual where tar was applied after discontinuing a series of ephedrine sulphate injections.

4. The results are discussed in relation to other work and certain concomitant conditions in cases of human cancer, and the opinion is expressed that carcinogenic agents act upon cells which have been deprived of a fully adequate vascular supply.

I have to thank Dr. N. Wood for looking after the mice during my absence from the laboratory, and Drs. I. Berenblum and G. M. Bonser, who allowed me to compare my controls with the records of their experiments with the same tar.

REFERENCES.

GULDBERG, G.—(1931) *Acta path. microbiol. Scand.*, Supp. viii.

JEANNENY, G.—(1932) "Cahiers de Radiologie," No. 7 (Supp. to No. 2, *Gaz. méd. de France*).

KREYBERG, L.—(1929) *Arch. path. Anat.*, **273**, 367.

STEWART, M. J.—(1931) *Lancet*, **2**, 565.

WARBURG, O.—(1930) 'The Metabolism of Tumours' (translated Dickens), London (Constable)

THE ACCURACY OF THE SKIN TEST IN CHINCHILLA RABBITS IN THE DETERMINATION OF THE STRENGTHS OF STREPTOCOCCAL TOXINS AND ANTITOXINS.

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VARIATION in the size of skin reactions produced by toxin injections is of two kinds: the " accidental variation " exhibited by an animal under identical injections, and due to such uncontrollable factors as blood-supply at the point of injection, depth of injection, etc., and " expected variation," due to changes in the strength of material injected. The reliability of any skin test depends in an inverse manner upon the magnitude of the " accidental variation ", and its delicacy depends directly upon the " expected variation ". The present investigation was carried out to determine the extent of these two variations.

The toxin used throughout this investigation was produced by Dick (1) and Dick (2) strains of hæmolytic streptococcus grown three days in beef infusion broth containing 1 per cent. peptone, 00·5 per cent. sodium chloride and 0·4 per cent. human blood. The toxicity was 40,000 skin test doses per c.c. (STD/c.c.) as determined on human subjects and on rabbits by standardization against toxin from the National Institute of Health, Washington. As a control formalinized streptococcal toxin was injected, in a dilution equal to the lowest toxin dilution, once into each rabbit on test. This formalinized toxin, containing 100 STD/c.c. in the undiluted state, was prepared by treating Dick (2) toxin with formalin, giving a final concentration of 0·5 per cent. at 37° C. for 10 months.

The backs of chinchilla rabbits (weighing 2 kg. or more) were closely clipped and marked off in squares of size approximately 3·5 cm. All injections were made posterior to the shoulders. The reactions produced by the intra-cutaneous injections of 0·1 c.c. of toxin dilution were measured in millimetres as the sum of two diameters at right angles to each other, 22–24 hours after injection. It is essential that readings be made in good daylight, but not in direct sunlight. It was thought that the intensity of the colour should contribute to the numerical measure of a reaction, and therefore the integers 0, 1, 2 and 3 were added according as the reaction was faintly pink, pink, intensely pink, or red. Since difference in intensity when present was generally associated positively with size of reaction, these numbers may be considered as crude approximations to a function of the size of the reaction. Small integers were chosen because changes in area rather than intensity of colour followed changes in the strength of the injections.

THE THEORETICAL DISTRIBUTION OF DIFFERENCES IN REACTIONS.

Each of 30 rabbits received from 7 to 16 (depending on size of back) injections of each of two dilutions of toxin, 1/250 (160 STD/c.c.) and 1/500 (80 STD/c.c.). Physiological saline was the diluent throughout the investigation. All injections in any one animal were made within a quarter of an hour. The dilutions were chosen in these strengths to avoid the complications incident to either maximal or minimal reactions. In all, 792 injections were made, of which 30 reactions were too diffuse to be accurately measured. These diffuse reactions were distributed about equally near the neck and tail. In the case of dilution 1/250 reactions near the hips tended to be larger than those near the neck. In fact, the average reaction for 50 injections was 2·5 mm. larger near the tail than the neck. For dilution 1/500 no such difference was evident. In any event, it is desirable to guard against this factor in any test by reversing the site of injection in one half of the rabbits.

The " accidental variation " was measured for our purposes by the standard deviation σ_i (for rabbit R_i) of reactions produced in a single animal by a set of identical injections which was estimated by means of the formula—

$$\sigma_i = \sqrt{\Sigma d^2/n-1},$$

where d represents the deviation of any reaction from the mean reaction for rabbit R_i and n is the number of reactions observed in that animal. Weighting each of these estimates (σ_i) according to its reliability (proportional to the square root of the number of observations), the mean standard deviation $\bar{\sigma}$ was calculated for each of the two dilutions, obtaining for dilution 1/250 $\bar{\sigma} =$ 2·493 with standard error $\sigma_{\bar{\sigma}}$ equal to 0·696 and for dilution 1/500 $\bar{\sigma} = 2{\cdot}180$ with standard error = 0·646. Assuming that both quantities are experimental estimates of the same quantity the difference is found to be just within the range of experimental error (less than 1·9599 × S.D. of difference), in which event the value 2·493 would equal or exceed the true value. To be on the safe side this value 2·493 was chosen as our final working estimate of the mean standard deviation of a reaction.

To carry out the analysis the assumption was made that the reactions in any one animal for identical injections were distributed normally. For obvious physical reasons it is impossible to test this assumption rigorously, but such a test as grouping the reactions exhibited by animals with the same standard deviations indicated that this is substantially the case.

Consider an animal R_i with standard deviation σ_i receiving one injection each of the standard (S) and the unknown (X) toxins producing reactions S_i and X_i respectively. Then the difference $d_i = S_i - X_i$ will be distributed normally with standard deviation $\sqrt{2\sigma_i^2}$ (Fisher, 1930, *a*). For a sample of n rabbits, the sum of the differences $D = \sum_{i=1}^{n} d_i$ will be distributed with standard deviation $\sqrt{2\Sigma\sigma_i^2}$. Assuming that the unknown and standard are of equal strength, this distribution will centre about the mean zero. Hence if $D > 1{\cdot}9599 \sqrt{2\sum_{i=1}^{n}\sigma_i^2}$, that is, if the deviation of the difference from the mean zero is greater than 1·9599 times the standard deviation (0·05 per cent.

level of significance [Fisher, 1930, *b*]), we conclude that this difference could not have arisen by chance from toxins of equal strength. Such a difference is called a significant difference.

To evaluate $\Sigma\sigma_i^2$ we write $\sigma_i = \bar{\sigma} + e_i$, so that e_i is the deviation from the mean. We have then—

$$\Sigma\sigma_i^2 = \sum_{i=1}^{n} (\bar{\sigma}^2 + 2\bar{\sigma}e_i + e_i^2)$$

$$= n\,(\bar{\sigma}^2 + \sigma_{\sigma}^2) + 2\,\bar{\sigma}\sum_{i=1}^{n} e_i.$$

The last term in the last expression representing the sum of the deviations about the mean will not be very different from zero, so that no great error is introduced by neglecting it and accepting as our working approximation—

$$\sqrt{\Sigma\sigma_i^2} = \sqrt{n(\bar{\sigma}^2 + \sigma_{\sigma}^2)} = 2{\cdot}5883\,\sqrt{n}.$$

Hence, for judgments of difference we must have—

$$D = \Sigma d_i > 1{\cdot}9599 \times 2{\cdot}5883\sqrt{2n}$$

$$> 5{\cdot}0728\,\sqrt{2n}.$$

If t injections of each toxin are made into each animal the standard deviation becomes $2{\cdot}5883\sqrt{2tn}$, with its corresponding level of significance $5{\cdot}0728\sqrt{2tn}$. It should be observed here that due to the neglect of the term Σe_i, of two tests involving an equal number of injections, that using more animals would be the more reliable.

For the more precise conception of a quantitative estimate, it is necessary to determine the functional relation between dose and reaction, or rather more precisely, the differences in the size of reactions due to differences in the strength of the injections—in other words, the "expected deviation" mentioned at the outset.

APPLICATION OF THE STANDARD DEVIATION.

To determine the "expected variation" and to check experimentally the practical application of the standard deviation derived in the first experiment, a second experiment was made with 64 rabbits, in which each animal received one injection each of 8 different toxin dilutions.

Here, owing to the occurrence of extremely diffuse margins arising from the weaker injections, it was found necessary to attempt to localize the reaction. Streptococcal antitoxin (400 Washington units per c.c.), when diluted 1/5000 and added to an equal volume of toxin, was found to have the desired effect of defining the margin of the reaction more clearly. Intravenous injection of 0·001 c.c./kg. streptococcal antitoxin (400 units per c.c.) tested in two rabbits likewise caused the margin of toxin reactions to be clearly defined. Other attempts at localization by the use of normal horse-serum, in dilutions varying from 1/50 to 1/500, 1·5 per cent. melted agar, gelatine in dilutions 1/10 to 1/1000 and tetanus antitoxin 1/5000, when mixed respectively with the toxin dilution failed to have the desired effect. The intravenous injection of trypan blue suggested by the work of Menkin (1929) was also unsuccessful in defining the margin of the reaction.

Diluting the toxin 1/100, 1/125, 1/156, etc., such that each dilution was 80 per cent. as strong as the preceding one and adding an equal volume of antitoxin (400 Washington units per c.c.) diluted 1/5000 to each dilution, a series of 8 dilutions was obtained. The syringes were then rinsed with 0·5 c.c. of the respective mixtures, filled, and arranged in an order unknown to the tester, thus eliminating any personal factor. Intradermal injection of 0·1 c.c. of each dilution was made once into each of 64 animals not previously tested for susceptibility to toxin. Thirty-five reacted to all the dilutions, 25 reacted to 2, 3 or 4, and 4 animals gave no reactions whatever. As before, the reactions were measured 22–24 hours after injection.

TABLE I.—*The Relation of Dose and Effect in Toxin Reactions.*

	Toxin dilution.							
	1:200.	1:250.	1:312.	1:390.	1:488.	1:610.	1:762.	1:952.
Skin test doses in each injection . . .	20	16	12·8	10·2	8·2	6·5	5·2	4·2
(*a*) Observed mean reaction (35 rabbits) . . .	34·6	32·7	30·5	28·6	27·1	26·7	24·9	23·1
(*b*) Calculated reaction ($R=12{\cdot}86-16{\cdot}26 \log D$) .	34·0	32·5	30·8	29·3	27·7	26·2	24·6	23·0
Calculated reaction ($R=16{\cdot}3\ D^{\frac{1}{4}}$) . . .	34·5	32·6	30·8	29·1	27·6	26·1	24·6	23·3
Crude differences, d . .		1·6	2·8	1·3	1·4	0·7	1·8	1·8
Smoothed differences, $\bar{d}$.		2·0	2·2	1·8	1·4	1·2	1·6	1·6

(*a*) Reactions were measured in millimetres as the sum of two perpendicular diameters plus a small integer depending upon the intensity of colour.
(*b*) R = size of reaction; D = skin test doses of toxin injected.

In Table I are listed the mean reactions for the various injections as calculated from the 35 more susceptible animals. In the fourth line we have the corresponding reactions for the best fitting logarithmic relation between dose and effect. The agreement is not very good. In the next row we have the best fitting curve of the form $R = bD^{\frac{1}{4}}$. Here the agreement is much better. From our point of view the fundamental quantities are not the mean reactions, which differ considerably from animal to animal, but the differences in mean reactions for the various doses. Row 6 gives these differences as calculated on the basis of all the animals giving reactions to two successive doses. Smoothing these crude differences (d) we derive the set $\bar{d}$, which was accepted for use in practical application.

On the basis of the differences $\bar{d}$ it is possible to forecast the number of correct discriminations between the various strengths of toxins. For, let the difference in mean reactions for the standard and unknown be $\bar{d}$, then, for a sample of n rabbits the difference in total reactions D will be distributed about the mean $n\bar{d}$ with standard deviation $2{\cdot}5883\sqrt{2n}$. In order that any particular difference may be significant, it is necessary that it exceed the level of

significance $L = 5{\cdot}0728\sqrt{2n}$). If we require, for instance, that correct distinctions be made in at least 95 per cent. of the cases, it is necessary (see table of probability integral) that—

$$\frac{n\bar{d}}{2{\cdot}5883\sqrt{2n}} - 1{\cdot}6449 \geqslant 1{\cdot}9599$$

or, that— $$n > 174/\bar{d}^2.$$

If t injections of each toxin are made into each animal, the formula becomes $n \geqslant 174t/\bar{d}^2$, where $\bar{d}$ represents the total difference for mean reactions of standard and unknown. This formula is very interesting, since given $\bar{d}$, the degree of precision required in estimation, it indicates at once the number of animals (or injections) to be used for the test, or, conversely, given the number of animals available it specifies the smallest difference which can be distinguished 19 times out of 20. (See Table II.)

TABLE II.—*Minimum Number of Rabbits Necessary to Distinguish in 95 per cent. of the Tests Various Differences in Strengths of Toxin and Antitoxin.*

Interval distinguished.	(1) *Toxin.* 2 injections of standard and 2 injections of unknown.	(2) *Antitoxin.* 3 injections of standard and 3 injections of unknown.
20 per cent.	22 rabbits.	11 rabbits.
36 ,,	7 ,,	3 ,,
50 ,,	4 ,,	2 ,,

(1) Standard toxin diluted to contain respectively 16 and 12·8 STD per injection.
(2) Standard antitoxin diluted to contain respectively 25/60, 20/60 and 16/60 Washington units per c.c. before addition to an equal volume of toxin.

TABLE III.—*Comparison of Observed and Theoretical Discriminations of Toxins of Different Strength.*

Strength of unknown. / Strength of standard.	Number of animals.	Number of tests.	Theoretical number of discriminations.	Observed number of discriminations.
0·80	4	75	11·6	20
0·80	6	51	10·7	11
0·80	30	7	4·8	6
0·64	4	60	28·2	33
0·64	5	51	28·7	26
0·64	20	6	5·9	6
0·50	5	41	34·5	31
0·50	6	34	30·5	30

One injection of unknown and of standard into each animal.

To test our ability to discriminate between toxins, the 60 animals which reacted to injections in the last experiment were divided into groups of 30, 20, 10, 6, 5 and 4, and the differences in total reactions compared with the corresponding levels of significance. Table III shows that the agreement between experiment and theory is remarkably good. Indeed it is so good as to arouse

suspicion. This agreement is to be expected, however, as the differences d were calculated from the observations themselves, so that in this sense the test is not an independent one. It does, however, indicate that the mean standard deviation as we have chosen it is a good approximation.

DISCRIMINATION BETWEEN ANTITOXINS.

An experiment to determine the strength of antitoxin by adding it to toxin and reducing the size of the resultant skin reaction, rather than completely neutralizing the toxin, resulted in even finer discriminations than in the toxin test.

The antitoxin was prepared from immune horse-serum, the horses having been injected with the Dochez (N.Y. 5) strain of hæmolytic streptococcus according to the method of Dochez (1924). The neutralizing power was 400 Washington units per cubic centimetre, each unit neutralizing 50 skin test doses of toxin. Five series of dilutions were prepared, the leading number of each series being 1/500, 1/625, 1/781, 1/977 and 1/1221 respectively, the remaining 4 members of each series being in geometric progression, each 80 per cent. as strong as the preceding one. Each dilution of antitoxin was added to an equal volume of toxin, diluted to contain 1000 STD/c.c., thoroughly mixed and allowed to stand at room temperature for an hour. One-tenth c.c. of each of the 25 dilutions of the toxin-antitoxin mixtures was then injected into each of 39 rabbits, and the measurements made and recorded precisely as in the preceding experiments. Since the factors inducing the " accidental variation " were the same as in the preceding tests and the reactions were due to the toxin present, the standard deviation used in the discrimination tests was that derived from the toxin reactions.

Thirty of the animals reacted to all but the mixture containing the lowest dilution (1/500) of antitoxin. These were divided into the various groups of 4, 5, 6, 10, 20 and 30, and the reactions for these groups tested for distinctions. Due to the presence of duplicate injections it was possible to test how many judgments of significant differences the method gave for identical injections. Series V (antitoxin dilutions 1/1221 to 1/2980) was omitted from these calculations, since, all the dilutions being injected in the region of the hips, it was not comparable to the remaining four series. The site of injection of the dilutions in the first four series, on the other hand, was reversed in one half of the animals. Using similar dilutions, the mean reaction for Series V was 1·6 mm. greater than for Series IV. Working with groups of 6 rabbits, there were in all 100 injections of duplicate dilutions, 6 of which were pronounced different. Theoretically the percentage of identical injections pronounced different should be 5 (0·05 per cent. level of significance).

Table IV shows the means for the various toxin-antitoxin reactions. Averaging differences between members of the same series, we get the crude differences (d) of the table. the smoothed differences ($\bar{d}$) occurring in the following line. In nearly all cases the smooth are smaller than the crude differences, so that the limits of error, assigned on the basis of these, would in all likelihood contain those based on the true differences. Table V shows the agreement between theoretical and experimental discriminations. The agreement is very

TABLE IV.—*The Relation of Dose and Effect in Toxin-Antitoxin Reaction.*

	Antitoxin dilution.								
	1 : 500.	1 : 625.	1 : 781.	1 : 977.	1 : 2211.	1 : 1526.	1 : 1907.	1 : 2354.	1 : 2980.
Washington units per c.c.	4/5	$(4/5)^2$	$(4/5)^3$	$(4/5)^4$	$(4/5)^5$	$(4/5)^6$	$(4/5)^7$	$(4/5)^8$	$(4/5)^9$
(1) *N* STD/c.c.	40·0	32·0	25·6	20·5	16·4	13·1	10·5	8·4	—
Observed mean reactions	19·18	23·50	25·80	28·81	31·23	33·49	35·27	36·90	—
(2) Calculated reaction $R = 61·74–25·70 \log D$	20·57	23·06	26·19	28·05	30·33	33·02	35·51	38·00	—
Crude differences, d		4·42	2·19	3·17	2·41	2·34	2·30	1·75	2·00
Smoothed differences, $\bar{d}$		3·5	3·0	2·8	2·5	2·4	2·2	2·0	1·8

(1) *N* STD/c.c., neutralizing skin test doses of antitoxin per c.c. before addition to an equal volume of toxin.
(2) R = Size of reaction ; D = neutralizing skin test doses of antitoxin per c.c. as above.

TABLE VI.—*Scale for the Determination of Differences in Strengths of Toxin and of Antitoxin.*

Strength of unknown. / Strength of standard.	1·95.	1·56.	1·25.	1·00.	0·80.	0·64.	0·51.	0·41.
(1) Toxin scale	..	8·00	4·20	0·00	—4·00	—7·20	—9·80	—12·60
(2) Antitoxin scale	—25·30	—16·00	—7·70	0·00	7·10	13·70	19·70	..

(1) Scale constructed for 2 injections of standard toxin containing 16 and 12/8STD respectively. Toxin of unknown strength diluted to contain presumably a similar number of STD.

(2) Scale constructed for 3 injections of standard toxin-antitoxin mixtures. Standard antitoxin diluted to contain respectively 25/60, 20/60 and 16/60 Washington units per c.c. before addition to an equal volume of toxin.

good, and indicates that the standard deviation as chosen is a good approximation.

TABLE V.—*Comparison of Observed and Theoretical Discriminations of Antitoxins of Different Strength.*

Strength of unknown. / Strength of standard.	Number of animals.	Number of injection in each animal.	Number of tests.	Theoretical number of discriminations.	Observed number of discriminations.
1·56	4	3	28	27·9	28
1·25	5	2	18	11·3	10
1·25	6	6	20	15·8	15
0·80	6	2	50	32·5	31
0·80	6	4	15	13·6	11
0·80	6	6	5	4·9	4
0·80	10	2	27	23·6	25

Column 3 indicates the number of injections of standard, the same number being used for the unknown.

PRACTICAL APPLICATIONS.

Toxin test.—Since it is desirable to take advantage of the large interval 2·2 (see $\bar{d}$, Table I), injections should be made in dilutions containing respectively 160 and 128 STD/c.c. For more than two injections of each toxin, multiples of this arrangement should be used, that is, for instance, two injections of the dilution containing 160 STD/c.c. and two injections of the dilution containing 128 STD/c.c. Table VI shows a scale for the determination of the difference in strengths of toxin. This scale, based on the differences $\bar{d}$ of Table I, was constructed in the following manner. Assume that the standard toxin of strength 40,000 STD/c.c. is injected once into each animal in dilutions 1/250 (160 STD/c.c.) and 1/312 (4/5 of 160 STD/c.c. = 128 STD) respectively. The toxin on test is injected in presumably the same strengths. If the unknown is 80 per cent. as strong as the standard, the difference between reactions of standard and unknown in dilution 1/250 should be — 2·2, and the difference due to dilution 1/312 should be — 1·8, giving a total difference of — 4·0 (negative sign because standard is stronger) per animal. Likewise, if the unknown is 64 per cent. as strong as the standard, the difference between reactions of standard and unknown should be — 7·2 (— 4 — 3·2). In a similar manner the other points were fixed on the scale. Intermediate points may be obtained by interpolation. A scale may thus be made to suit any set of injections, the one given in Table VI being applicable, of course, only for the two dilutions used in its construction.

In a test with n animals, each animal receiving $2t$ injections (t sets of 2 injections) of each toxin, the difference (D) in total reactions (unknown toxin minus standard toxin) should be compared with $L = 5{\cdot}0728 \times \sqrt{4\,nt}$. If D is greater than L we conclude that the unknown is different from the standard. To determine the interval containing the unknown, we find $D \pm L$. These

two bounds should then be divided by nt to obtain the variation for a single set of injections in a single animal and the corresponding limits found by interpolation in Table VI.

As an application, consider the results of a test with 10 rabbits in which each animal received one injection of 0·1 c.c. of each of two dilutions of toxin containing 160 and 128 STD/c.c. respectively, and presumably similar injections of the unknown toxin. The difference (D) in total reactions was — 57. The standard deviation for such a sample is, by our formula, $2{\cdot}5883\sqrt{40} = 16{\cdot}368$. The deviation is therefore, on the assumption of equality, $57/16{\cdot}368 = 3{\cdot}48$ standard deviations. The probability of such a difference occurring by chance, from injections of the same strength of toxin, is less than one in a thousand, so that the toxins are certainly different. To estimate the extent of this difference we have that the true reaction for the unknown would lie (95 times out of 100) within the bounds $-57 \pm L = -57 \pm 1{\cdot}9599 \times 16{\cdot}368 = -57 \pm 32{\cdot}079$, or for a single animal the limits are — 2·5 and — 8·9. Referring to Table VI we find by interpolation that—

$$0{\cdot}875 < \frac{\text{Unknown}}{\text{Standard}} > 0{\cdot}555.$$

Actually the ratio unknown/standard was 0·640.

Antitoxin.—To test the strength of antitoxin, 3 dilutions of the standard should be made to contain respectively 16/60, 20/60 and 25/60 Washington units per cubic centimetre. If the strength of undiluted antitoxin is known to be 400 units the dilutions should be 1/900, 1/1200 and 1/1500 respectively. The unknown should be diluted to contain presumably the same amounts of antitoxin. Each antitoxin dilution is then added to an equal volume of toxin diluted to contain 1000 STD/c.c.

As an application we considered the results of a test where each of 4 animals received one injection of each toxin-antitoxin mixture, the three dilutions being respectively 1/900, 1/1200 and 1/1500 before mixing with an equal volume of toxin. Both standard and unknown were injected in equal dilutions. The difference (D) in total reactions was — 57. The standard deviation for such a sample is $2{\cdot}5883\sqrt{24} = 12{\cdot}6826$, and the level of significance $L = 24{\cdot}851$. Since D is greater than L, the unknown differs from the standard. To determine the limits we obtain $-57 \pm 24{\cdot}851$, giving the two bounds — 32·149 and — 81·851. Dividing by 4, the number of animals, we find that the limits are — 8 and — 20·4. From Table VI, scale for antitoxin, we conclude that—

$$1{\cdot}744 < \frac{\text{Unknown}}{\text{Standard}} > 1{\cdot}261.$$

Actually the ratio was 1·56.

The reduction of the size of a reaction is a much more delicate test for the strength of antitoxin than a test dependent upon complete neutralization. This may be seen by comparing the results given in this paper with those given in Table II of Fraser and Plummer (1930).

The mathematical treatment found in this paper was contributed by S. A. Lischinsky, Ph.D.

SUMMARY.

1. The standard deviation of a streptococcal toxin reaction in an individual rabbit was calculated to be 2·493 mm. with standard error 0·696.

2. Reactions produced by streptococcal toxin mixed with an equal volume of dilute streptococcal antitoxin have more sharply defined margins than those produced by toxin mixed with a similar volume of saline, tetanus antitoxin, gelatine, normal horse-serum or agar.

3. A test for the strength of streptococcal antitoxin based upon reduction in size of toxin-antitoxin reaction rather than upon complete neutralization has been outlined.

4. Assuming that the two toxins are differentiated if correct distinctions could be made 95 times out of 100, it is possible on the basis of the present experiment to distinguish a 20 per cent. difference in the strength of two toxins with a test involving 22 rabbits, 2 injections of each toxin into each animal. In the case of antitoxin, using 3 injections of each antitoxin, 11 animals are sufficient. Similarly 4 rabbits are required to distinguish a 50 per cent. difference in the strengths of two toxins, and 2 rabbits suffice to distinguish a 50 per cent. difference in antitoxins.

REFERENCES.

DOCHEZ, A. R.—(1924) *Trans. Ass. Amer. Phys.*, **39**, 136.
FISHER, R. A.—(1930, *a*) 'Statistical Methods for Research Workers', London (Oliver & Boyd), p. 101.—(1930, *b*) *Ibid.*, p. 77.
FRASER, F. H., AND PLUMMER, H.—(1930) *Brit. J. Exp. Path.*, **11**, 291.
MENKIN, V.—(1929) *J. Exp. Med.*, **50**, 171.

AN ATTEMPT TO PRODUCE IMMUNITY TO INDUCED TUMOURS IN MICE.

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As long ago as 1906 it was shown by Bashford, Murray and Cramer (1906–1907) that the injection of mouse blood into a mouse induces resistance against the inoculation of a tumour; and in the same year Schöne (1906) proved that the injection of mouse embryo emulsion has the same effect. This discovery was quickly extended by Borrel (1907), Bridré (1907), Bashford, Murray and Haaland (1908) and others, and it was shown that, although various normal tissues could induce resistance to tumour-inoculations, embryo skin was the most effective. In 1912 Higuchi (1912) confirmed much of the previous work, and emphasized in particular the specific nature of the action of various tissues.

From a summary of more recent work, to be found in Woglom's (1929) valuable paper, it appears that no essentially new facts concerning this immunity have been discovered, and embryo skin remains the most efficient homologous tissue in inducing resistance, as well as being the most convenient to obtain sterile. Heterologous tissue rarely produces immunity (indeed, in some instances, it has been found to cause hypersensitivity), but opinions vary on this subject. From the various contradictory results he reports, Woglom decides that "the only justifiable conclusion to be drawn from an experiment is that the material used for immunization produced resistance in the strain of animals employed, against the tumour injected for the testing inoculation, at the time when the experiment was done".

The immunity produced in this way is believed to be of the same type as that produced naturally by the absorption of an inoculated tumour, and Russell (1908) concluded that the inoculation of embryo tissue prevents the provision of a vascular supply for any subsequent tumour inoculation. Russell found, however, that although mice which had already received one tumour inoculation could be to some extent immunized against a second inoculation if an injection of embryo skin was given previously to the latter, the formation of metastases from inoculated tumours was not prevented by skin-injections, nor could a tumour-graft which had already commenced growth be made to regress by this means.

It is known that the injection of embryo skin causes no immunity against the development of spontaneous tumours. At the time when the following experiments were begun, in June, 1924, it was still unknown what effect injections of embryo skin would have upon the development of tumours induced by tar-painting. Accordingly these experiments were designed to find out whether it was possible in this way to produce an immunity to such induced tumours, *i. e.* whether the appearance of such tumours would be merely delayed, or would be entirely prevented.

For reasons which will be mentioned later, the results were not published at the time, and, in 1927, there appeared a paper by Fibiger and Moeller (1927, *a*, *b*) dealing with the very same question. It may be said at once that the results presented here are similar to those obtained by Fibiger and Moeller.

METHODS.

Beginning in June, 1924, 100 mice (50 does and 50 bucks) were tarred once a week (or, occasionally, more often) until definite skin-changes were seen. These were the controls for the experiments. The tar used was ethereal solution of gas tar, previously extracted with water and with methylated spirits, the residue from one pint of tar being dissolved in 26 oz. of ether; this solution was later (after two or three weeks) diluted by the addition of an equal quantity of ether and used at this strength for subsequent paintings.

At the same time, another 100 mice (48 does and 52 bucks) were painted twice at weekly intervals with the strong solution (and a third time with the weak solution, for those which did not show complete epilation). Then, a fortnight later, by which time all the hair had re-grown, some of the mice were inoculated with 0·5 c.c. of a saline emulsion of mouse embryo skin;

the rest of the mice received their first inoculation 6 weeks or 2 months after the cessation of tarring. Tarring was not resumed until August or September, approximately one month after the inoculation of skin, and was continued once a week or oftener, as in the controls, until gross changes were seen in the skin.

As in the experiment of Fibiger and Moeller, the mice received various numbers of inoculations of embryo skin ; 31 bucks received two inoculations and 21 received nine, while 24 does received five, 12 received seven and 12 received eight. The intervals between the inoculations also varied from one, two or three months to (towards the end of the experiment) a fortnight or three weeks. Most of the inoculations were done with 0·5 c.c. of skin per mouse, but this was increased to ·75 c.c., and finally to 1 c.c. for the last two or three inoculations. The last inoculation was given 268 days after the recommencement of tarring.

The times of appearance of the skin changes were counted from the recommencement of tarring in the inoculated animals and from the first tarring in June in the controls.

RESULTS.

The results of the experiment are shown in the accompanying tables. For purposes of comparison, and to serve as a second control, the results of a later tar-painting experiment are also given ; this experiment was begun in 1929 with mice which belonged to a pure non-cancerous albino strain, obtained originally in 1927 from Prof. F. E. Crew, of Edinburgh, and bred for several generations in this laboratory.

Table I shows the numbers of mice surviving at the end of each month, and the total numbers of tumours which had appeared by that time. It will be seen that the survival rate is poor in the inoculated mice, and not really good in the controls, but is better in the 1929 mice.

The actual numbers of tumours appearing in each successive month are given in Table Ia.

In Table II the total numbers of tumours are expressed as a percentage of the surviving mice in each month. This method masks the poor survival rate, but shows perhaps more clearly than Table I that the inoculations had no effect upon the development of tumours ; this is confirmed by the results of the 1929 experiment.

In Table III a detailed analysis of the results is given. Apart from the earlier appearance of the first wart in the inoculated mice, the skin changes appear at approximately the same time in the three sets of mice. The total numbers of tumours agree quite well, considering the survival rate in each set ; it will be noticed that, although most of the tumours were epitheliomata, a few sarcomata were found—one in the case of the inoculated mice, one in the 1929 set, and four in the 1924 controls.

There was no noticeable difference between the sexes, as regards numbers of tumours or times of their development. In the 1929 experiment, until the last month, there were fewer tumours in the does than in the bucks in comparison with the numbers of surviving mice, but in the 1924 experiment

TABLE I.—*Showing Actual Numbers of Survivors and Tumours in Inoculated and Control Mice at End of Each Month from Time of First Application of Tar, with Figures from Later Tar Experiment for Comparison.*

Months.	1924–25.												1929–30.					
	Inoculated.						Control.						Pure strain.					
	Males.		Females.		Both sexes.		Males.		Females.		Both sexes.		Males.		Females.		Total.	
	S.	T.	S.	T.	S.	T.	S.	T.	S.	T.	S.	T.	S.	T.	S.	T.	S.	T.
1	46	..	44	..	90	..	50	..	49	..	99	..	48	..	50	..	98	..
2	46	..	42	..	88	..	48	..	48	..	98	..	47	..	49	..	96	..
3	39	..	40	..	79	..	47	..	43	..	90	..	47	..	49	..	96	..
4	38	..	39	..	77	..	45	..	40	1	85	1	47	..	49	..	96	..
5	32	1	36	1	68	2	45	*2	39	2	84	4	46	..	49	2	95	2
6	29	2	33	6	62	8	42	4	38	*8	80	12	45	5	49	4	94	9
7	22	9	30	11	52	20	41	11	37	*22	78	33	43	18	46	12	89	30
8	18	11	30	17	48	28	39	17	37	28	76	45	40	*24	43	25	83	49
9	18	14	27	*24	45	38	33	20	36	30	69	50	39	34	43	30	82	64
10	17	15	27	26	44	41	30	*25	36	30	66	55	38	37	43	33	81	70
11	17	15	27	27	44	42	27	25	34	33	61	58	38	37	41	36	79	73
12	16	16	27	27	43	43	27	25	34	34	61	59	37	37	37	37	74	74
13	..	..	..	..	..	..	25	25	34	34	59	59	..	..	..	..	..	..

S. = Survivors. T. = Tumours.

TABLE Ia.—*Showing Numbers of Tumours Appearing During Each Month of the Experiment. Pure Strain for Comparison.*

Year.		Months.								
		4.	5.	6.	7.	8.	9.	10.	11.	12.
1924 to 1925	Inoculated	..	2	6	12	8	*10	3	1	1
	Control	1	*3	*8	*21	12	*5	5	3	1
1929 to 1930	Pure strain	..	2	7	21	*19	15	6	3	1

* These figures include 1 sarcoma in each case.

* These figures include 1 sarcoma in each case.

TABLE II.—*Showing Percentage of Mice with Tumours for each Successive Month from First Application of Tar until the End of the Experiment. Figures from Later Tar Experiment for Comparison.*

Months.	1924–25.						1929–30		
	Inoculated.			Control.			Pure strain.		
	Males.	Females.	Total.	Males.	Females.	Total.	Males.	Females.	Total.
3	..	..	..	..	..	..	..	..	..
4	..	..	..	..	2·5	1·25	..	..	..
5	3·1	2·5	2·95	*4·4	5·1	4·75	..	4·0	2·0
6	7·0	18·0	12·5	9·5	*21·0	15·25	11·0	8·1	9·5
7	40·9	36·7	38·8	26·8	*59·4	43·1	42·0	26·0	34·0
8	61·0	56·7	58·85	46·0	75·7	60·85	*60·0	58·1	59·0
9	77·7	*88·9	83·3	60·6	83·3	71·95	87·2	69·8	78·5
10	88·2	96·0	92·1	*83·3	83·3	83·3	97·0	76·7	86·9
11	82·2	100·0	94·1	92·6	97·0	94·8	97·0	87·8	92·4
12	100·0	100·0	100·0	92·6	100·0	96·3	97·0	100·0	98·5

* These figures include 1 sarcoma in each case.

TABLE III.—*Detailed Analysis of Results of Embryo Skin Inoculation Experiment with Results of a Later Tar Experiment for Comparison. Time in Months from First Application of Tar.*

	1924–25.						1929–30.		
	Inoculated.			Control.			Pure strain.		
	Males.	Females.	Total.	Males.	Females.	Total.	Males.	Females.	Total.
Average time for appearance of warts	5	5	5	6	8	7	5	6	5·5
,, ,, ,, epitheliomata	7	8	7·5	8	8	8	8	8	8
Appearance of first wart	1·5	3	1·5	3	4	3	4	4	4
,, ,, epithelioma	4·5	5	4·5	5	4	4	6	5	5
,, ,, sarcoma	..	9	9	5	6	5	8	..	8
Longest-lived non-cancerous mouse	12	9	12	13	11	13	12	13	13
Total number of warts	24	32	56	37	34	71	43	41	84
,, ,, epitheliomata	16	26	42	23	32	55	36	37	73
,, ,, sarcomata	..	1	1	2	2	4	1	..	1
,, ,, tumours	16	27	43	25	34	59	37	37	74
Number of gland metastases (epithelioma)	2	3	5	5	4	9	12	6	18
,, ,, ,, (sarcoma)	..	..	..	..	1	1	..	..	..
,, ,, ,, (total)	2	3	5	5	5	10	12	6	18
,, lung metastases (epithelioma)	..	..	..	4	..	4	8	4	12

there were relatively more tumours in the control does than in the control bucks, and in the inoculated mice there was no real difference.

In the last part of Table III there are shown the numbers of metastases in the three sets of mice. Only the metastases which were visible macroscopically were taken into account, and there are, therefore, comparatively few compared with the numbers given by Fibiger and Moeller (who examined for microscopical metastases as a matter of routine). But twice as many gland metastases (10) were found in the control mice than in the inoculated (5), although the number of tumours (59) in the controls was not twice that in the inoculated mice (43); moreover, there were 4 lung metastases in the control mice, and none in the inoculated. (In the 1929 experiment, on the other hand, the numbers of gland and lung metastases were much greater [18 and 12 respectively], but too much importance need not be placed on this fact, as these mice cannot be regarded as strict controls for the first experiment.)

Although these figures are, perhaps, not so satisfactory as the greater numbers given by Fibiger and Moeller, they point to the same conclusion, namely, that, although the inoculation with embryo skin had no inhibitory effect upon the appearance of primary skin tar-tumours, it did seem to reduce the numbers of metastases formed by these tumours. This is all the more striking, since the conditions of the two experiments were not identical; Fibiger and Moeller tarred their animals more frequently and for a much shorter period, and the intervals between the skin inoculations were much less than in the experiment described here; also, they did not continue the inoculations for so long, their last one being 137 days after the first tarring.

The preliminary tarring received by the animals in this experiment may be ignored, as having no effect on the skin, since the hair had completely re-grown at the time of the first inoculation.

In some cases the inoculations were continued after the appearance of a tumour in an attempt to immunize the mouse and cause regression of the tumour, but the attempt was a complete failure. This confirms the results previously obtained by Russell.

The results described above were not published at the time, for several reasons. In the first place, there was found to be a great individual variation in the type of growth, its time of appearance and rate of development; and in the second place, the mice, although all about the same age, and all obtained from the same breeder, did not belong to one strain and the colours varied. It was questionable whether these differences would affect the value of the result, although the control mice had the same origin and showed the same individual variations as regards skin changes.

The mice in the second tar-painting experiment in 1929 showed the same individual variation, however, in spite of the fact that they belonged to a pure strain; these mice received weekly paintings with tar, but no inoculations of embryo skin.

Further, a tumour which appeared in this second experiment was transplanted into mice belonging to the pure strain and into mice of the same origin as those used in 1924, and both sets gave similar results.

Accordingly it may be concluded that the variations did not detract from the value of the results of this 1924 experiment.

In 1929 certain experiments were begun to test the heredity of the response to tar-painting. The mice which developed the earliest tumours were bred from, and it was intended to tar their young, select again from these the mice developing the earliest tumours, and so build up, if possible, a strain of mice showing an early response to tar-painting. Unfortunately, before any definite result had been obtained, the experiments had to be abandoned on account of lack of space.

SUMMARY.

1. The development of tar-tumours was neither prevented nor delayed in mice which had received various numbers of inoculations with mouse embryo skin. Accordingly, the injection of skin has no protective action against the genesis of an induced tumour.

2. Inoculations of mouse embryo skin continued after the appearance of tar-tumours had no curative effect, and the tumours did not regress.

3. The numbers of metastases found in the control mice were so much greater than those found in the inoculated mice that it is suggested that the inoculations of skin, although useless for producing an immunity to induced tumours, do develop a resistance in the animal against the metastatic spread of malignant cells.

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REFERENCES.

Bashford, E. F., Murray, J. A., and Cramer, W.—(1906) *Brit. Med. J.*, **2**, 207.—(1907) *Proc. Roy. Soc.*, B, **79**, 164.

Bashford, E. F., Murray, J. A., and Haaland, M.—(1908) *Third Sci. Rep. Imp. Canc. Res. Fund*, p. 359.

Borrel, A.—(1907) *Bull. Inst. Pasteur*, **5**, 497, 545, 593, 641.

Bridré, J.—(1907) *Ann. Inst. Pasteur*, **21**, 760.

Fibiger, J., and Moeller, P.—(1927, *a*) *C. R. Soc. Biol.*, **96**, 1463 —(1927, *b*) *Lancet*, **1**, 1248 (abstract).

Higuchi, S.—(1912) *Fifth Sci. Rep. Imp. Canc. Res. Fund*, p. 79.

Russell, B. R. G.—(1908) *Third Sci. Rep. Imp. Canc. Res. Fund*, p. 341.—(1912) *Fifth Sci. Rep. Imp. Canc. Res. Fund*, p. 1.

Schöne, G.—(1906) *Münch. med. Wschr.*, **53**, 2517.

Woglom, W. H.—(1929) "Immunity to transplantable tumours", *Cancer Rev.*, **4**, 129.

THE "FIXED" VIRUS OF RABIES: THE ANTIGENIC VALUE OF THE VIRUS INACTIVATED BY THE PHOTODYNAMIC ACTION OF METHYLENE-BLUE AND PROFLAVINE.

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PERDRAU and Todd (1933, *a*) confirmed and extended Clifton's (1931) observations on the sensitiveness of certain bacteriophages to the photodynamic action of methylene-blue. They also showed that some animal viruses, viz. those of vaccinia, herpes, fowl plague, louping ill, Borna disease and canine distemper are also highly sensitive to irradiation by visible light in the presence of this dye (Perdrau and Todd, 1933, *b*). The fact that the virus of distemper after inactivation in this way preserved its antigenic properties and could be used as an efficient vaccine (Perdrau and Todd, 1933, *c*) awakened a new interest in the phenomenon. It was decided therefore to study the photodynamic action of methylene-blue and other dyes on the virus of rabies and to determine whether the virus so inactivated had any antigenic value; the results obtained in protecting rabbits against "fixed" virus form the subject of this paper.

EXPERIMENTAL METHODS.

(a) *Strain of Virus.*

The strain of "fixed" virus which has been used in these experiments is that of the Pasteur Institute in Paris. This strain was chosen since it was reported to be capable of infecting rabbits when inoculated by the intramuscular route. This method of infection is less severe than the cerebral one, and therefore more applicable to immunity tests. Further, this strain is believed to possess good immunizing properties (Cunningham, Malone and Craighead, 1933). It is now generally recognized that strains of "fixed" virus may differ, both as regards their power to produce infection when introduced by routes other than the intracerebral, and also as regards their antigenic properties (Balozet, 1932–33; Remlinger and Bailly, 1931; Cunningham, Malone and Craighead, 1933).

(b) *Dyes and Conditions of Irradiation.*

Experiments have been made with both methylene-blue* (Grübler) and proflavine.† The final concentration of the dyes used was 1 : 50,000. In those experiments made with unfiltered virus suspensions, equal parts of a 1 : 25,000 solution of the dye and of the virus suspension were mixed, but when collodion membrane or sand and paper pulp filtrate were employed, 1 c.c. of a 1 : 10,000 solution of the dye in distilled water or physiological saline was mixed with 4 c.c. of the virus filtrate.

The conditions of irradiation have been kept constant, and only the time factor has varied. The mixture of virus and dye was exposed in a layer 2 mm. deep in a Petri dish placed 13 cm. below a 300 c.p. filament lamp. Overheating of the virus was avoided by the interposition of a glass vessel through which cold water was running continuously, and by suitable control of inlet and outlet flow a depth of 2 in. of water was maintained between the source of light and the exposed virus.

* Methylenblau (Koch) für Bazillenfärbung (Grübler).

† A small sample kindly supplied by the British Dyestuffs Corporation.

The Petri dishes containing the virus-dye mixtures were placed over a water-jacket, through which cold water was also running. The upper surface of this jacket is a polished plate of aluminium—a good conductor of heat. These additional precautions were also of service in obviating overheating.

Controls included (*a*) a mixture of the virus and dye kept in the dark during the period of the experiment, and (*b*) distilled water mixed with the virus suspension or filtrate and exposed to the same source of light as the mixtures of virus and dye.

The time of exposure to the source of light varied from 15 minutes to one hour. In each test the mixtures were exposed in duplicate for the same period of time, and one rabbit was inoculated intracerebrally with a 0·25 c.c. sample of each mixture.

(c) *Virus Preparations.*

(1) *Unfiltered suspensions of virus* were employed in the early experiments. Portions of the brain of a rabbit, killed when showing advanced symptoms about the 9th day after intracerebral inoculation, were ground in a mortar with powdered glass, and adding sufficient broth to give the required concentration. This suspension was centrifugalized for 10 minutes at 2500 revs. per minute; the supernatant fluid was pipetted off and stood at 4° C. for 24 hours to allow of further sedimentation. The final supernatant fluid was used for the experiment.

(2) *Collodion membrane filtrate.*—Concurrently with the present experiments, a study was being made in collaboration with Dr. W. J. Elford on the filterability of the virus, so that active permeable membrane filtrates of fixed virus were also available. One experiment was made with such a filtrate.

(3) *Sand and paper pulp filtrates.*—Five per cent. centrifugalized suspensions in broth of virulent brain were filtered through standardized sand and paper pulp filters. Filtrates prepared in this way were relatively clear, and had an infectivity of approximately one-tenth that of a centrifugalized unfiltered suspension.

Photodynamic Action of Methylene-Blue and Proflavine on the Virus.

For the purpose of simplicity in the exposition of the results, experiments made with methylene-blue and proflavine will be grouped together, although all the early experiments were made with methylene-blue. It will be recalled that Herzberg (1931) showed that in addition to methylene-blue several acridine dyes were capable of inactivating the virus of vaccinia in the presence of light. The present writer made some experiments on the virus of foot-and-mouth disease, which is resistant to the photodynamic action of methylene-blue (Perdrau and Todd, 1933, *b*), and found that this virus was relatively sensitive to inactivation by certain acridine dyes, notably proflavine. In association with this work, Burnet (1933) also found that proflavine was relatively more active on certain phages than methylene-blue.

Experiments with Unfiltered Suspensions of Virus.

In two experiments made with methylene-blue the brains from which the virulent suspension were prepared had been stored in glycerine for periods of 20 and 35 days respectively. It was considered advisable to use virus material stored in this way in preference to fresh material in order to minimize any possible "protection" of the virus by living cells (Perdrau and Todd, 1933, *b*). The conditions of irradiation were as described above, and the results of the two experiments are given in Table I. These results show that under the conditions of the experiments no inactivation of "fixed" rabies virus in unfiltered virus suspensions occurred. For this reason it was decided to attempt inactivation of the virus as it is contained in collodion membrane filtrates, or in sand and paper pulp filtrates. It is, however, convenient at this stage to

refer briefly to later experiments in which a comparison was made of the photodynamic action of proflavine and methylene-blue. Two experiments were made. The time of exposure of the mixtures to irradiation was one hour,

TABLE I.—*Irradiation of Unfiltered Glycerinated Virus Suspensions with Methylene-Blue.*

Date of experiment.	No. of rabbit.	Time of irradiation.	Concentration of dye in mixture.	Concentration of virus in mixture.	Result of inoculation.
(1) 30.3.33	PV635	15 mins.	1 : 50,000	1·25% (brain stored in glycerine at 4° C. for 20 days)	Died of rabies.
	PV636				,, ,,
	PV637	30 ,,	1 : 50,000		Survived.
	PV638				Died of rabies.
	PV639	60 ,,	1 : 50,000		,, ,,
	PV640				,, ,,
	PV641 (control)	Kept in dark	1 : 50,000		,, ,,
	PV642 (,,)	60 mins.	No dye		,, ,,
	PV643 (,,)	Virus not irradiated	,,		,, ,,
	PV646 (,,)				,, ,,
(2) 28.4.33	PV689	15 mins.	1 : 50,000	0·5% (brain stored in glycerine at 4° C. for 35 days)	Died of rabies.
	PV690				,, ,,
	PV687	30 ,,	1 : 50,000		64th day.
	PV688				Died of rabies.
	PV685	60 ,,	1 : 50,000		,, ,,
	PV686				,, ,,
	PV692 (control)	Kept in dark	1 : 50,000		,, ,,
	PV691 (,,)	60 mins.	No dye		,, ,,

and in other respects the conditions obtaining were as in the experiments described above, except that fresh virulent brain was used for preparing the suspension instead of one preserved in glycerine. The results of the two experiments are recorded in Table II.

TABLE II.—*Comparison of Photodynamic Action of Methylene-Blue and Proflavine on Unfiltered Virus Suspensions.*

Date of experiment.	No. of rabbit.	Dye used.	Virus concentration in mixture.	Result of inoculation.
(1) 22.9.33	PV898	Proflavine	1·25%	Survived.
	PV897	Methylene-blue		Died of rabies.
	PV900 (control)	Proflavine in dark		,, ,,
(2) 4.10.33	PV908	Proflavine	1·25%	Survived.
	PV907	Methylene-blue		Died of rabies.
	PV903 (control)	Proflavine in dark		,, ,,

These results suggest that the "fixed" virus of rabies is relatively more sensitive to the photodynamic action of proflavine than to that of methylene-blue, at least in the presence of physiologically active cells, or portions of cells

from an infected animal. No evidence of "protection" of the virus against inactivation by the proflavine in the presence of light was obtained under the conditions of the experiments.

Experiment with a Collodion Membrane Filtrate.

One experiment was made (Table III). It showed definitely that it is possible to inactivate a virulent collodion membrane filtrate by the photodynamic action of methylene-blue. However, as it was found that the titre of an active filtrate prepared in this way was relatively low, it was decided to make some experiments with tissue-free fitrates prepared by filtration of a virus suspension through standardized sand and paper pulp filters.

TABLE III.—*Inactivation of Fixed Rabies Virus in a Collodion Membrane Filtrate by Light and Methylene-Blue.*

(Average pore diameter of membrane, 0·8μ.)

Date of experiment.	No. of rabbit.	Time of irradiation.	Concentration of methylene-blue.	Result of inoculation.
11.4.33	PV657	15 mins.	1 : 50,000	Survived.
	PV658			
	PV655	30 ,,	1 : 50,000	,,
	PV656			
	PV653	60 ,,	1 : 50,000	,,
	PV654			
	PV652 (control)	Mixture kept in dark	1 : 50,000	Died of rabies.
	PV651 (control)	60 mins.	No dye	,, ,,

Experiments with Sand and Paper Pulp Filtrates.

The results of an experiment made with such a filtrate are given in Table IV.

TABLE IV.—*Inactivation of Fixed Rabies Virus in a Sand and Pulp Filtrate by Light and Methylene-Blue* (9.5.33).

No. of rabbit inoculated.	Time of irradiation.	Concentration of dye in mixture.	Result of inoculation.
PV700	15 mins.	1 : 50,000	Survived.
PV701			
PV702	30 ,,	1 : 50,000	,,
PV703			
PV704	60 ,,	1 : 50,000	,,
PV705			
PV714 (control)	Mixture kept in dark	1 : 50,000	Died of rabies.
PV713 (control)	60 mins.	No dye	,, ,,

They show that it was possible to inactivate the virus suspended in a sand and paper pulp filtrate by the photodynamic action of methylene-blue. Similar results were obtained with proflavine.

It might also be mentioned that in the immunity experiments, the results of which are about to be described, each batch of irradiated virus administered to rabbits was also inoculated intracerebrally to other rabbits to prove that the virus was definitely inactivated. Fourteen different samples of virus and methylene-blue which had been irradiated for one hour and used as vaccine were tested in this way. Six similar irradiated mixtures of proflavine and virus were likewise tested. In no case was evidence obtained that the virus was not inactivated.

Antigenic Value of Virus after Photodynamic Inactivation by Methylene-Blue or Proflavine.

In these experiments inactivated mixtures of sand and paper pulp filtrates of virus and methylene-blue or proflavine were administered to rabbits by intramuscular injection, with the object of determining whether they possessed any antigenic value. The reason for using sand and paper pulp filtrates in preference to collodion membrane filtrates was that the former contained a relatively greater amount of virus as determined by titration experiments. The time of irradiation to ensure inactivation of the virus was one hour. Three experiments were made with virus inactivated by the photodynamic action of methylene-blue, and one with virus inactivated by the photodynamic action of proflavine. The results obtained in the two cases were very similar. The number of doses of vaccine administered varied in the different experiments. These doses were given intramuscularly into the lumbar region, and the test dose of virus in the form of a sand and paper pulp filtrate was also given by this route.

The results of the four experiments are given in Tables V, VI, VII and VIII.

TABLE V.—*Antigenic Value of Virus after Inactivation by the Photodynamic Action of Methylene-Blue.*

No. of rabbit.	Doses of vaccine. 9.5.33.	23.5.33.	2.6.33.	Test dose (13.6.33).	Result.
PV715	10 c.c.	4 c.c.	4 c.c.	2 c.c. S.P.F. of virus intramuscularly 11 days after last dose of vaccine	Died of rabies.
PV716					Survived.
PV717					,,
PV654*					,,
PV655*					,,
PV657*					,,
PV776	Controls intramuscular inoculation of virus				Died of rabies.
PV777					,, ,,
PV786	Control intracerebral inoculation			0·25 c.c.	,, ,,

* *N.B.*—These three rabbits had previously had a dose of irradiated virus intracerebrally.

S.P.F. = Sand and paper pulp filtrate.

TABLE VI.—*Antigenic Value of Virus after Inactivation by the Photodynamic Action of Methylene-Blue.*

No. of rabbit.		Doses of vaccine. 6.7.33.	8.7.33.	10.7.33.	Test dose (21.7.33).	Result.
PV831*	3 doses.	10 c.c.	10 c.c.	10 c.c.	2 c.c. S.P.F. of virus intramuscularly 11 days after last dose of vaccine	Died of rabies 27th day.
PV832	3 doses.	10 c.c.	10 c.c.	10 c.c.		Survived.
PV833	3 doses.	10 c.c.	10 c.c.	10 c.c.		,,
PV834	3 doses.	10 c.c.	10 c.c.	10 c.c.		,,
PV797	2 doses.	None	10 c.c.	10 c.c.		,,
PV820	2 doses.	None	10 c.c.	10 c.c.		,,
PV835*	2 doses.	None	10 c.c.	10 c.c.		,,
PV836	2 doses.	None	10 c.c.	10 c.c.		,,
PV839	2 doses.	None	10 c.c.	10 c.c.		,,
PV838	2 doses.	None	10 c.c.	10 c.c.		,,
PV824	1 dose.	None	None	10 c.c.		Died of rabies.
PV840*	1 dose.	None	None	10 c.c.		,, ,,
PV837	1 dose.	None	None	10 c.c.		Survived.
PV841	1 dose.	None	None	10 c.c.		Died of rabies.
PV792	Controls intramuscular inoculation of virus					,, ,,
PV842	Controls intramuscular inoculation of virus					,, ,,
PV763	Controls intramuscular inoculation of virus					,, ,,
PV812	Controls intramuscular inoculation of virus					,, ,,
PV843	Control intracerebral inoculation				0·25 c.c.	Died of rabies.

* *N.B.*—These three rabbits had previously had a dose of irradiated virus intracerebrally.
S.P.F. = Sand and paper pulp filtrate.

TABLE VII.—*Antigenic Value of Virus after Inactivation by the Photodynamic Action of Methylene-Blue.*

No. of rabbit.	Doses of vaccine.	Test dose (13.9.33).	Result.
PV881			Survived.
PV879	2 c.c. 22.8.33		,,
PV873	2 c.c. 24.8.33		,,
PV872	2 c.c. 26.8.33		,,
PV875	2 c.c. 28.8.33		Died of rabies.
PV877	3 c.c. 30.8.33	2 c.c. S.P.F. of virus intramuscularly 7 days after last dose of vaccine	Survived.
PV878	4 c.c. 1.9.33		,,
PV880	4 c.c. 4.9.33		,,
PV871	4 c.c. 6.9.33		,,
PV874			Died of rabies.
PV891	Controls		,, ,,
PV892	Controls		,, ,,
PV893	Controls		,, ,,
PV894	Controls		,, ,,
PV895	Controls		,, ,,
PV896	Controls		,, ,,
PV897	Control	0·25 c.c. intracerebrally	,, ,,

TABLE VIII.—*Antigenic Value of Virus after Inactivation by the Photodynamic Action of Proflavine.*

No. of rabbit.	Doses of vaccine. 19.10.33.	25.10.33.	31.10.33.	Test dose (15.11.33).	Result.
PV909	5 c.c.	4 c.c.	4 c.c.	2 c.c. S.P.F. of virus intramuscularly 15 days after last dose of vaccine	Survived.
PV910					,,
PV911					Died of rabies.
PV915					Survived.
PV916					,,
PV927	Controls				Died of rabies.
PV928					Symptoms, but recovered.
PV929					Died of rabies.
PV930					,, ,,
PV920	Control			0·25 c.c. intracerebrally	,, ,,

The results show that of 35 rabbits which had received doses of "fixed" virus inactivated by the photodynamic action of methylene-blue or proflavine, and which were subsequently tested by intramuscular injection of living virus, 27 survived and 8 died of rabies, *i. e.* survival rate of 77 per cent. If one excluded the 4 rabbits in Table VI which only received one dose of virus the rate of survival among vaccinated rabbits is 84 per cent. Of the 16 control rabbits inoculated intramuscularly with living virus, 15 died of rabies and only 1 survived, *i. e.* a percentage of survival about 6 per cent.

Two other tests may be reported here: (1) Rabbits PV716, PV717, PV654, PV655, PV657 (*vide* Table V), which had survived the intramuscular test dose of virus given on June 13th, 1933, were later inoculated intracerebrally (on July 21st, 1933, *i. e.* 38 days later) with 0·25 c.c. of a sand and paper pulp filtrate of "fixed" virus (S.P.F.). All of these died of rabies. (2) Rabbits PV653, PV656 and PV658 (*vide* Table III), which had received a small dose of inactivated virus intracerebrally on April 11th, 1933, were inoculated intramuscularly on May 23rd, 1933 (*i. e.* 42 days later), with 2 c.c. of S.P.F. virus, and all three died of rabies. The first of these two experiments shows that resistance to an intramuscular test dose does not connote cerebral immunity.

DISCUSSION.

It has been found possible to inactivate the "fixed" virus of rabies as it exists in collodion membrane or sand and paper pulp filtrates, but not in unfiltered virus suspensions of glycerinated fresh rabbit brain or brain stored in glycerine by the photodynamic action of methylene-blue under the conditions of our experiments. On the other hand, the virus even in unfiltered suspensions is sensitive to the photodynamic action of proflavine. This may be due to the greater penetrating power of this dye.

It is possible that if irradiation of unfiltered virus suspensions in the presence of methylene-blue were more prolonged, or the source of light more intense,

inactivation might be obtained in this case also. Since no titrations were made of the irradiated mixtures of virus suspensions and methylene-blue, the possibility cannot be excluded that some degree of inactivation less than absolute had been effected. In work of this nature the concentration of virus in the suspensions subjected to irradiation may influence greatly the results. The superiority of proflavine over methylene-blue in inactivating the virus of rabies under the action of light in the presence of physiologically active cells or portions of cells from an infected animal may be only very relative. Further experiments would be necessary to determine this point.

Sufficient suggestive evidence, however, has been obtained to indicate that, even in experiments with sand and paper pulp filtrates, in which there is always the possibility, however remote, of an occasional cell or cell particle getting into the filtrate, it is probably safer to employ proflavine rather than methylene-blue.

As regards the immunity experiments, the results show that the "fixed" virus of rabies inactivated by the photodynamic action of methylene-blue or proflavine preserves its antigenic value, since rabbits injected with it were able to resist infection with the same virus introduced intramuscularly. More than one dose of the vaccine, however, appears to be necessary to produce this result. The fact that rabbits which had received doses of inactivated virus and subsequently living virus intramuscularly succumbed to an intracerebral infection administered some time later merits some consideration. It is well known how difficult it is to immunize against a neurotropic virus, such as that of rabies, unless living virus be given, and it is the opinion of many that immunity of the central nervous system cannot be effected unless the living virus reaches this tissue. If this be so, it appears likely that the living fixed virus given intramuscularly to rabbits which had previously received inactivated virus never reached the central nervous system, as the rabbits did not prove later to be immune to an intracerebral inoculation of virus.

SUMMARY.

1. The "fixed" virus of rabies is sensitive to the photodynamic action of methylene-blue. Under the conditions of the experiments it was inactivated in collodion membrane or sand and paper pulp filtrates, but not in unfiltered virus suspensions.*

2. The virus appears to be relatively more sensitive to the photodynamic action of proflavine than to that of methylene-blue, at least in the presence of physiologically active cells or portions of cells from an infected animal. It was inactivated by irradiation when the former dye was employed even in unfiltered virus suspensions of fresh infected brain.

3. Rabies fixed virus inactivated by the photodynamic action of methylene-blue or proflavine conserves its antigenic potency, since about 84 per cent.—26 out of 31 rabbits—which had received more than one dose of such a vaccine

* Our attention has been drawn to a paper in the January, 1934, number of the *Indian Journal of Medical Research*, which has just reached us, by Shortt, H. E, and Brooks, A. G., on the photodynamic action of methylene-blue on fixed rabies virus. They used daylight as a source of light, and succeeded in demonstrating the sensitiveness of the virus in unfiltered virus suspensions of fresh brain to the photodynamic action of methylene-blue.

survived an intramuscular test dose of fresh virus, while of 16 unvaccinated control animals only 1 survived (percentage survival, 6).

I would like to express my thanks to Drs. Perdrau and Todd for much useful help and criticism during the course of these experiments.

REFERENCES.

BALOZET, L.—(1932–33) *Arch. Inst. Past.*, Tunis, **21**, 130.
BURNET, F. M. —(1933) *J. Path. Bact.*, **37**, 179.
CLIFTON, C. E.—(1930–31) *Proc. Soc. Exp. Biol. Med.*, **28**, 745.
CUNNINGHAM, J., MALONE, R. H., and CRAIGHEAD, A. C.—(1933) *Ind. Med. Res. Memoirs*, No. 26 (Calcutta).
HERZBERG, K.—(1931) *Zbl. Bakt.*, I., Orig. **122**, 231.
PERDRAU, J. R., AND TODD, C.—(1933, *a*) *Proc. Roy. Soc.*, B, **112**, 277.—(1933, *b*) *Ibid.*, **112**, 288.—(1933, *c*) *J. Comp. Path.*, **46**, 78.
REMLINGER, P., AND BAILLY, J.—(1931) *Ann. Inst. Past.*, **47**, 608.
SHORTT, H. E., AND BROOKS, A. G.—(1934) *Ind. J. Med. Res.*, **21**, 581.

THE PROPAGATION OF THE VIRUS OF VESICULAR STOMATITIS IN THE CHORIO-ALLANTOIC MEMBRANE OF THE DEVELOPING HEN'S EGG.*

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IN a previous paper (Galloway and Elford, 1933) it was recorded that the results of filtration experiments with standardized collodion filter membranes had shown that the virus particles of the virus of vesicular stomatitis of horses and cattle were very much larger than those of the virus of foot-and-mouth disease. This was regarded as direct evidence of the distinct individuality of the two diseases. Reference was also made to the fact that experiments had been made by the present writers, which demonstrated that the virus of vesicular stomatitis will survive and propagate in the chorio-allantoic membrane of the developing hen's egg, and that the virus of foot-and-mouth disease, on the other hand, had been found not to survive under similar conditions.

The purpose of the present note is to give the results of experiments which demonstrate that the virus of vesicular stomatitis can be propagated in the chorio-allantoic membrane of the developing hen's egg, and further to describe the types of reaction produced, and indicate to what extent the method is useful in the study of the virus.

* This work was done on behalf of the Foot-and-Mouth Disease Research Committee, who have kindly given their permission for the publication of this paper.

EXPERIMENTAL METHODS.

Strain of virus.—Both immunological types of the virus have been employed in these experiments—the "Indiana" and the "New Jersey". Primary passages to eggs were made with broth collodion membrane filtrates.

Technique of inoculation eggs.—The technique of inoculation was based on that described by Woodruff and Goodpasture (1931). Slight modifications have been introduced from time to time, and these have been described for the most part in previous papers (Burnet, 1933, and Burnet and Ferry, 1934).

Briefly the method is as follows: 9- to 10-day-old embryos are employed. The position of the natural air-sac is marked. A triangular piece of shell is removed from the side of the egg, the opening being effected with a cutting circular disc fitted to a dental engine. A small hole is drilled through the shell into the air-sac. A slit is made with a needle through the shell membrane exposed after removal of the triangular piece of shell. The most recent modification is at this stage. In order to avoid injury to the chorio-allantoic membrane when attempting to raise the sides of the slit to produce the formation of an artificial air-sac between shell membrane and chorio-allantoic membrane, slight suction is applied to the opening into the natural air-sac. This facilitates the separation of the chorio-allantoic from the shell membrane. The inoculum is deposited on the surface of the chorio-allantoic membrane at the base of the artificial air-sac. A rim of paraffin-vaseline is formed round the triangular opening, and on this a sterile coverslip is placed. The hole over the air-sac is also closed with a drop of paraffin vaseline. The eggs are incubated at 39° C., and examined daily by transillumination.

EXPERIMENTAL FINDINGS.

It has been found that both the immunologically different strains of virus, "Indiana" and "New Jersey", can be propagated in the egg without difficulty. The "Indiana" strain has undergone ten successive passages without showing any signs of diminished pathogenicity for guinea-pigs or eggs, and fifteen have been made with "New Jersey" strain. The changes produced in the eggs have shown rather a sharp distinction into two groups. The majority of the embryos of the eggs which have received an inoculation of virus have died between 24 and 48 hours after inoculation, without showing any characteristic lesions beyond slight œdema with occasional hæmorrhage of the chorio-allantoic membrane. The other type of response has a closer resemblance to, but is generally less intense, than that encountered at this site in similar experiments with some other viruses, such as that of canary-pox. The membrane shows regularly distributed patches, often very small, of ectodermal proliferation, and a varying degree of inflammatory reaction, œdema, opacities and new blood-vessel formation. Of the ten passages of the Indiana strain mentioned above the sequence of lethal (L) and non-lethal (NL) types of change has been NL→NL→NL→L→L→NL→L→L→L→L. This would suggest an increase in the pathogenicity of the virus for the embryo with successive passages, but, on the other hand, many direct inoculations to eggs of guinea-pig virus filtrates have given the lethal type of reaction. Of 6 "Indiana" strain filtrates prepared from guinea-pig lesions, 4 killed the embryo within 48 hours and 2 gave non-lethal reactions, while 3 out of 4 similar tests made with the "New Jersey" strain have given the non-lethal type of reaction. Up to the present, no success has been attained in efforts to determine the factor or factors responsible for deciding which type of reaction may result. The "Indiana" strain appears to be more prone to give lethal reaction than the "New Jersey" strain, and in two comparative experiments in which

the batch of eggs and conditions of incubation were identical, the former gave lethal and the latter non-lethal reactions. In most experiments, however, this strain also has given lethal reactions similar to those obtained with the " Indiana " strain. Occasionally it has been observed that more concentrated virus killed the embryo while the highest dilutions gave non-lethal reactions, but it is more usual to find the same type of response through the whole series of dilutions.

Distribution of the Virus through the Egg.

This question has not been studied in detail, but sufficient experiments have been made to justify the statement that when the lethal effect is produced, the virus is present in moderate to high concentration in the chorio-allantoic membrane and in the brain, skin and liver of the embryo. In testing organs for the presence of virus, the embryo was well washed with sterile water and approximately 0·1 gm. quantities of tissue ground up in 5 c.c. of broth. Such suspensions of tissue were tested for virus by inoculation of guinea-pigs. Inoculations were made intradermally into the pads and also subcutaneously. Dilutions of the 2 per cent. suspension were also tested by inoculation of eggs by the usual method. The results of the egg inoculations, which were further confirmed by sub-inoculation into guinea-pigs, were that the membrane suspensions always infected at a dilution of 1 : 10,000, but not often at a higher titre. Of three suspensions of brain tested from embryos killed by the virus, two were infective at 1 : 10,000 (*i. e.* 1 : 500,000 of tissue), and one at 1 : 100. Skin and liver from the one embryo examined both infected at 1 : 10,000.

With regard to the non-lethal response results have been less regular, and the virus seems to be much less constantly present in the embryo. Four embryo brain suspensions were tested without dilution, of which two only contained virus. The membrane lesions themselves also appear to contain less virus, at least at certain stages, than is usual with eggs that have been killed by the virus. Owing to the impossibility of arranging conditions under which non-lethal lesions should regularly occur, we have been obliged to take only occasional opportunities to investigate this point. One series of three eggs was inoculated with a 1 : 100 dilution of a filtrate of " Indiana " virus which had been stored for some time in the ice-chest. Eggs were opened on the 2nd, 4th and 6th day, and an emulsion of each membrane inoculated into guinea-pigs. All three eggs showed membrane lesions. That of the egg opened on the 6th day, however, although it did not show any definite epithelial proliferation, showed considerable thickening and irregular opacities. The guinea-pigs inoculated with 2- and 4-day lesions reacted typically, while the two inoculated with 6-day membrane failed to do so. When reinoculated with virus later these last two guinea-pigs did not prove to be immune. On two other occasions typical membrane lesions removed on the 3rd and 4th day and inoculated into two guinea-pigs each gave a delayed reaction in one of the guinea-pigs and nothing in the other. In at least one other instance an egg showing the usual appearances of a positive lesion failed to infect either of the two guinea-pigs in which the subinoculation was made. It seems reasonable to conclude that if conditions are such as to prevent the early lethal action of the virus in 24 to 48 hours, the inflammatory and proliferative reaction of the membrane is largely

effective in preventing the virus from infecting the embryo, and in some cases at least may destroy the virus in the chorio-allantoic membrane itself.

Histology of the Membrane Lesions.

The chorio-allantoic membrane lesions conform to the general type of virus lesion in this tissue and no individually characteristic feature can be recorded. A moderate degree of ectodermal proliferation seems to represent the primary lesion. This seldom gives rise to layers more than 3 or 4 cells thick. The ectodermal cells soon become degenerate with " ballooning " and necrosis, and at the 3rd day the ectodermal layer nearly always contains many leucocytes and red blood-cells, usually in a fragmented state. No " inclusion bodies " have been observed, though fragmented red cells within ectodermal or endodermal cells might sometimes easily be mistaken for them. The mesodermal layer shows the usual highly variable inflammatory changes, œdema, new blood-vessel formation and leucocytic or fibroblastic cellular infiltration. The endodermal epithelium proliferates to a moderate degree without any other characteristic changes.

Immune Serum Neutralization of Virus: Comparative Inoculation Tests on Eggs and Guinea-pigs.

Three experiments have been made. The immune sera employed were collected from guinea-pigs which had been proved immune to a homologous strain of virus about one month after the original infection with the " New Jersey " or " Indiana " strain of virus.

The method of carrying out the serum neutralization tests was to mix equal parts of the immune serum with falling dilutions of a collodion membrane virus filtrate in broth. The mixtures were left for 30 minutes at 15° C.–20° C., and then samples were removed for inoculation into guinea-pigs and also into eggs. The first experiment was made with a virus filtrate in broth of the " Indiana " strain of virus obtained from guinea-pig pad lesions and an homologous guinea-pig immune serum. The results of this experiment are given in Table I. They show quite definitely that the egg-inoculation technique can be employed for testing the virus-neutralizing properties of an immune serum, but that a virus-serum mixture (undiluted virus and immune serum—*vide* Table I), which may produce no reaction when inoculated into guinea-pigs, may produce a response in the egg, and that virus can be recovered from the egg lesion so produced by subinoculation into guinea-pigs. The other two experiments in this series were made to determine (1) if the results of immune serum neutralization tests were similar irrespective of whether guinea-pig or " egg " virus was used, and (2) whether the egg inoculation technique could be employed to prove the immunological difference between the " Indiana " and " New Jersey " strains of virus. The guinea-pig virus employed in the first of these two experiments was a collodion membrane filtrate in broth. The " egg " virus was a broth-suspension prepared from an infected chorio-allantoic membrane by grinding in a mortar and centrifugalizing. Both strains of virus were employed, and mixtures made with both homologous and heterologous sera. The methods of making the mixtures and test inoculations into eggs and guinea-pigs were the same as in the experiment described above.

TABLE I.—*Immune Serum Neutralization of Virus: Comparative Test Inoculations in Eggs and Guinea-pigs.*

"Indiana" Guinea-pig virus—homologous immune serum.

Inoculations.	Broth + dilutions of virus.					Immune serum + dilutions of virus.					Remarks.
	Undiluted.	1 : 10.	1 : 100.	1 : 1000.	1 : 10,000.	Undiluted.	1 : 10.	1 : 100.	1 : 1000.	1 : 10,000.	
Guinea-pigs	++	++	++	+0	00	00	00	00	00	00	Two guinea-pigs inoculated with each mixture.
Eggs	+	+	+	+	+	+	0	0	0	0	One egg inoculated with each mixture.
Control inoculation of guinea-pigs from eggs	—	—	—	—	++	++	0	0	—	—	Two guinea-pigs inoculated with each egg.

TABLE II.—*Immune Serum Neutralization of Virus: Comparative Test Inoculations in Eggs and Guinea-pigs.*

Guinea-pig virus—homologous and heterologous sera.

Inoculations.	Broth + dilutions of virus.				Immune serum + dilutions of virus.							
	"New Jersey" strain (N.J.).		"Indiana" strain (Ind.).		N.J. virus + N.J. serum.		N.J. virus + Ind. serum.		Ind. virus + Ind. serum.		Ind. virus + N.J. serum.	
	1 : 100.	1 : 10,000.	1 : 100.	1 : 10,000.	1 : 100.	1 : 10,000.	1 : 100.	1 : 10,000.	1 : 100.	1 : 10,000.	1 : 100.	1 : 10,000.
Guinea-pigs	++	00	++	00	00	00	++	+0	00	00	++	00
Eggs	+	+	+	+	0	0	+	+	0	0	+	+
Control inoculation of guinea-pigs from eggs	—	+0	—	++	00	00	—	++	00	00	—	++

— = No test made.

TABLE III.—*Immune Serum Neutralization of Virus: Comparative Test Inoculations in Eggs and Guinea-pigs.*

Egg virus—homologous and heterologous sera.

Inoculations.	Broth + dilutions of virus.				Immune serum + dilutions of virus.									
	"New Jersey" strain (N.J.).		"Indiana" strain (Ind.).		N.J. virus + N.J. serum.		N.J. virus + Ind. serum.		Ind. virus + Ind. serum.		Ind. virus + N.J. serum.	
	1 : 100.	1 : 10,000.	1 : 100.	1 : 10,000.	1 : 100.	1 : 10,000.	1 : 100.	1 : 10,000.	1 : 100.	1 : 10,000.	1 : 100.	1 : 10,000.
Guinea-pigs	++	00	++	++	00	00	+0	00	00	00	++	++
Eggs	+	+	+	+	0	0	+	+	+	0	+	+
Control inoculation of guinea-pigs from eggs	—	++	—	—	00	00	—	++	++	00	—	—

TABLE IV.—*Comparative Titrations of Virus Filtrates in Eggs and Guinea-pigs.*

Filtrates.	Inoculations.	Dilutions of virus.						
		1 : 10.	1 : 100.	1 : 1000.	1 : 10,000.	1 : 100,000.	1 : 1,000,000.	1 : 10,000,000.
(1)	Guinea-pig	—	++	+0	00	00	—	—
	Egg	—	+	+	+	+	+*	0
(2)	Guinea-pig	—	++	+0	+0	00	00	00
	Egg	—	+	+	+	+	+*	0
(3)	Guinea-pig	++	+0	00	00	—	—	—
	Egg	+	+	+	+*	—	—	—
(4)	Guinea-pig	—	++	+0	00	00	—	—
	Egg	—	+	+	+	+*	—	—
(5)	Guinea-pig	++	+0	00	00	—	—	—
	Egg	+	+	+	+*	—	—	—
(6)	Guinea-pig	—	++	00	00	00	—	—
	Egg	—	+	+	+	+*	0	—

— = No test made.

* Control subinoculation to guinea-pigs from these eggs gave positive results.

The results are given in Tables II and III. They show quite definitely that it is possible to employ the egg to prove the immunological identity of the two different strains of virus, and also that the results are very similar, whether the guinea-pig or egg virus is used. The results of one of the tests (Table III : Indiana virus + Indiana serum 1 : 100) was similar to that already observed and described above, viz. that an immune serum-virus mixture may produce no reaction in guinea-pigs by inoculation, but give lesions in eggs, from which virus can be recovered by subinoculation into guinea-pigs. Further, it will be observed from the results of the titrations of virus in these experiments that a higher dilution infected eggs than guinea-pigs, and that in general the egg is much more sensitive to small doses of virus than the guinea-pig.

Other observations have since been made on numerous occasions which have brought ample confirmation to the existence of this interesting phenomenon, and in Table IV the results of comparative titrations in eggs and guinea-pigs of different freshly prepared or stored filtrates are recorded.

Photodynamic Inactivation by Methylene-Blue.

Experiments had previously been made by one of us (I. A. G.), which showed that under the standard conditions described below the virus of vesicular stomatitis in broth filtrates was sensitive to inactivation by methylene-blue in the presence of light. About one hour appeared to be the time required to obtain complete inactivation of an undiluted filtrate having a titre as determined by guinea-pig inoculation of about 1 : 10,000. When the results referred to in the previous section were obtained, that the egg was more sensitive to the virus than the guinea-pig, it was decided to make an experiment to determine whether by inoculation into eggs any trace of non-inactivated virus could be detected in irradiated mixtures giving no reactions when inoculated into guinea-pigs.

The mixtures of virus and dye were exposed in a layer 2 mm. deep in Petri dishes placed 13 cm. below a 300 c.p. filament lamp. The final concentration of the dye in the mixtures was 1 : 50,000. Overheating of the virus was avoided by the interposition of a glass vessel through which cold water was running continuously, and by suitable control of inlet and outlet flow a depth of 2 in. of water was maintained between the source of light and the exposed virus. The Petri dishes containing the virus-dye mixtures were placed over a water jacket, through which cold water was also running. The upper surface of this jacket was a polished plate of aluminium—a good conductor of heat. These additional precautions were also of service in obviating overheating. The mixtures were irradiated for from 30 minutes to 2 hours, and samples were inoculated into eggs and guinea-pigs. The results of the experiments are given in Table V.

TABLE V.

Time of irradiation.	Guinea-pig test.	Egg test.	Control sub-inoculation of guinea-pigs from eggs.
30 mins.	++	+	++
60 ,,	00	0	00
2 hours	00	0	00

+ = Virus present.

They show that the tests made on eggs and guinea-pigs gave similar results, and no evidence was obtained in this one experiment that an irradiated mixture which produced no reactions in guinea-pigs contained a trace of virus which could be detected by the egg inoculation technique. A point of some interest which is being further investigated from the point of view of vaccinating animals was that the four guinea-pigs which had been inoculated intradermally into the pads with samples of the mixtures inactivated by irradiation for one hour and two hours later proved to be solidly immune to infection with the virus.

DISCUSSION.

It has been found possible to propagate the virus of vesicular stomatitis in the chorio-allantoic membrane of the developing hen's egg. This is not surprising when one takes into consideration the fact that Cox, Syverton and Olitsky (1933) showed that the virus could be propagated in tissue cultures of chick embryo. It may also be recalled that Maitland and Maitland (1931) failed to cultivate the virus of foot-and-mouth in similar tissue cultures. The usefulness of the method for the study of the virus has been clearly demonstrated. The fact that the inoculation of eggs renders possible the detection of doses of virus which produce no reaction by direct inoculation into guinea-pigs is of importance. It suggests that a re-examination should be made of results of filtration experiments previously reported on, to determine whether filtrates giving negative reactions in guinea-pigs might not be shown still to contain some virus by the egg inoculation technique. This is being done, and the results will be described in a later paper.

It is not easy to find an explanation of the phenomenon that a dose of virus which produces no lesion in the guinea-pig may produce lesions in eggs from which virus can be recovered by subinoculation to guinea-pigs, but it may be recalled that it is relatively easy to produce immunity to vesicular stomatitis in guinea-pigs, and that subinfective doses of virus will often do this. In the egg the rate of multiplication of the virus is probably very rapid and no immunity response comes into the picture.

SUMMARY.

(1) Both the " Indiana " and " New Jersey " strains of virus have been propagated in the chorio-allantoic membrane of developing hen's eggs.

(2) The types of reactions produced in inoculated eggs are described as well as the histology of the lesions.

(3) In certain cases the virus may be recovered not only from the chorio-allantoic membrane, but also from the brain, skin and liver of the embryo.

(4) It has been found possible to employ the egg inoculation technique for the testing of immune sera, and determining the identity of the two immunologically different strains of virus.

(5) Doses of virus which produce no reactions in guinea-pigs may produce lesions in eggs from which virus can be recovered by subinoculation into guinea-pigs.

(6) The virus is relatively sensitive to the photodynamic action of methylene-blue. Irradiated mixtures which give no lesions when inoculated into guinea-pigs are also inactivated for eggs.

We wish to express our thanks to Miss G. H. Faulkner for making some passages in eggs of the New Jersey strain of virus.

REFERENCES.

BURNET, F. M.—(1933) *J. Path. Bact.*, **37**, 107.
Idem AND FERRY, J. D.—(1934) *Brit. J. Exp. Path.*, **15**, 56.
COX, H. R., SYVERTON, J. J., AND OLITSKY, P. K.—(1933) *Proc. Soc. Exp. Biol. Med.*, **30**, 896.
GALLOWAY, I. A., AND ELFORD, W. J.—(1933) *Brit. J. Exp. Path.*, **14**, 400.
MAITLAND AND MAITLAND.—(1931) 'Fourth Progress Report Foot-and-Mouth Disease Research Committee,' London (H.M. Stationery Office).
WOODRUFF, A. M., AND GOODPASTURE, E. W.—(1931) *Amer. J. Path.*, **7**, 209.

IMMUNIZING FRACTIONS ISOLATED FROM *BACT. AERTRYCKE.*

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THE studies recorded in this and the two following papers are concerned with the immunizing and toxic activities of various fractions isolated from *Bact. aertrycke.* Though not directly related in their inception, they have converged during their later stages. As will be seen, the different activities studied appear to be referable, in large part at least, either to a single chemical substance, or to substances that are closely associated in the various chemical fractions that have so far been obtained. The present report deals with attempts that we have made during the past twelve months to isolate from *Bact. aertrycke* a fraction capable of inducing active immunity in mice.

The Antigenically Active Components of Bacterial Cells.

It is impossible to review in any detail the numerous studies that have followed the isolation by Heidelberger and Avery (1923) of type-specific polysaccharides from pneumococci, and by Zinsser and Parker (1923) of similar components from a variety of bacterial species. It is sufficient to note that the majority of these studies have suggested that the bacterial polysaccharides,

when isolated in a state of chemical purity, have the characters of haptens in the sense defined by Landsteiner. They react specifically in the test-tube with antisera prepared by the injection of the corresponding bacterial cells, but do not themselves stimulate antibody-production when injected into the animal tissues. They have, for the most part, been reported to be non-toxic.

Other constituents of the bacterial cell, for instance the bacterial proteins, are fully antigenic, inducing in the living animal the formation of antibodies with which they react *in vitro*.

Turning to the problem of active immunization, we find a considerable body of evidence indicating that effective antibacterial immunity depends, in large part at least, on the presence of antibodies acting on the antigens that characterize the surface of a bacterium in its virulent smooth form (Avery and Morgan, 1925; Avery and Neill, 1925; Avery and Goebel, 1931; Felix, 1924; Arkwright, 1927; Ibrahim and Schutze, 1928; Robertson and Felix, 1930; Greenwood, Topley and Wilson, 1931; Henderson, 1932; Grinnell, 1932, and others). Many of these surface components are complex polysaccharides. In the particular group to which *Bact. aertrycke* belongs the nature and behaviour of these surface somatic antigens have been demonstrated by the work of Furth and Landsteiner (1928, 1929), White (1929, 1931), Meyer (1931), Combiesco, Stamatesco and Soru (1930) and others.

The view that has until recently seemed to accord best with all the available evidence has been that the specific somatic polysaccharides, which are of major importance in the induction of active immunity, are dependent for full antigenic activity on their linkage to some other component of the bacterial cell, possibly a protein, and that this link is broken by the methods of chemical isolation employed. Since the isolated polysaccharides have proved inactive *in vivo*, we have had to rely, for purposes of active immunization, on crude suspensions or extracts of bacterial cells, with all their attendant disadvantages.

It is true that observations are on record suggesting that polysaccharides, or polysaccharide-containing fractions, prepared from pneumococci may induce an active immunity (Perlzweig and Steffen, 1923; Perlzweig and Keefer, 1925; Schiemann and Casper, 1927; Schiemann, Lowenthal and Hackenthal, 1931; Wadsworth and Brown, 1931, 1933; Felton, 1932), but the exact relationship of these immunizing fractions to the specific polysaccharide has, in the past, been far from clear, and the possibility remained, in most cases at least, that their activity depended on the presence of traces of an undisrupted antigenic complex of an undetermined nature.

In the particular case of *Bact. aertrycke*, Casper (1928) recorded the isolation, by solution in antiformin followed by precipitation with alcohol, of a polysaccharide substance that was toxic for mice and induced some degree of active immunity. The degree of resistance attained was, however, of very doubtful significance. Of 39 immunized mice tested in the two experiments recorded, 37 died of the infection within 13 days, as against 16 of 17 controls. The only difference between the immunized and control mice was that a few of the former lived rather longer than the latter. Ten of the immunized mice were tested for the presence of agglutinins, all with negative results.

Very recently, when most of the experiments outlined in this report had been completed, Avery and Goebel (1933) recorded their observations on the

immunizing properties of the acetylated form of the Type I pneumococcal polysaccharide, and Boivin and his colleagues (1933, 1934) described the preparation and properties of a toxic and immunizing fraction isolated from *Bact. aertrycke* and from other organisms. Since these observations have a direct bearing on our own results, they will most conveniently be considered when these have been described.

EXPERIMENTAL PROCEDURE.

The Method of Immunological Assay.

Each of the fractions that we have isolated from *Bact. aertrycke* has been compared, as regards its immunizing potency, with an arbitrarily selected standard preparation of unfractionated bacterial cells, an additional control group of non-immunized mice being included in each test. The standard employed has been a preparation of dried bacterial cells, killed and partially extracted with acetone, and stored in the cold over calcium chloride. It has shown no loss of activity during the ten months that it has been in use. Similar preparations have formed the starting material for all fractionations.

In preliminary trials it was found that 0·017 mgm. of the standard preparation formed a convenient immunizing dose. This corresponds approximately to 5×10^7 bacilli. A dose of this size is almost non-toxic, and induces a significant but relatively slight increase in resistance, as judged by a lengthening in the mean time to death of those mice that die after the test inoculation of living bacilli, or by the percentage of survivors on the 28th day. Two injections of this dose, at a week's interval, are followed by a slightly greater increase in resistance. Over a large series of tests normal mice, injected in groups of 30 with 1000 *Bact. aertrycke* of the virulent strain used throughout these experiments, have shown 0–6·67 per cent. of survivors, and a mean time to death of about 5–7 days. Mice immunized with the standard preparation have shown 10–30 per cent. of survivors, and a mean time to death of about 12–15 days. A partial immunity of this degree has formed a convenient standard against which to measure the potency of our fractions, since it has enabled us to detect differences in either direction.

The experiments with which we are here concerned have been carried out as follows :

Thirty-four mice have been injected intraperitoneally with 0·017 mgm. of the fraction under test dissolved or suspended in 0·5 c.c. of sterile Ringer's solution, and the same number of mice have been injected with the same dose of the standard preparation. Several different fractions have usually been tested at the same time, each on 34 mice, this number being selected to allow for possible deaths during the immunization period. One week later all injections have been repeated in the same dose and by the same route. One week after the second injection 30 mice from each of the immunized groups and 30 normal controls have been injected intraperitoneally with 1000 living *Bact. aertrycke.* All mice have been housed in separate cages and observed for 28 days. All dead mice have been examined bacteriologically. In almost every case *Bact. aertrycke* has been isolated.

The Results of Preliminary Experiments.

No useful purpose would be served by giving detailed protocols of our earlier experiments. It will suffice to note that bacterial bodies fully extracted in sequence with ethyl ether, petroleum ether, chloroform and methyl alcohol, as well as with acetone, retained their potency ; that acetone-extracted bacilli brought into solution by digestion with trypsin, after the method adopted by Douglas and Fleming (1923) and Douglas (1923), but continued to the point of maximal hydrolysis, were as active as the standard preparation ; and that the precipitate obtained from such a digest by the addition of 68 per cent. alcohol, after a preliminary clearing by centrifugation, proved rather more active than the standard preparation.

The fraction precipitated by increasing the alcohol concentration to 95 per cent. after separation of the 68 per cent. precipitate, and the alcohol-soluble fraction prepared by evaporating the residual alcoholic solution *in vacuo*, were quite inactive.

Our further experiments have so far been confined to a preliminary refractionation and testing of the alcohol-precipitable immunizing substance.

The Method of Preparation of the Immunizing Fractions.

Roux bottles containing ordinary nutrient agar are sown, in batches of 40–50, with a smooth virulent strain of *Bact. aertrycke*. After 24 hours' incubation at 37° C. the growth from each bottle is washed off in 10 c.c. of sterile tap-water. The washings are combined in large sterile centrifuge tubes and to them is added an equal volume of acetone. After standing for about an hour at room temperature the tubes are centrifuged and the supernatant fluid is removed and discarded. Each tube is now about half filled with pure acetone, in which the deposited bacteria are thoroughly emulsified, and incubated at 37° C. overnight. Next morning the bacteria are found flocculated and deposited with a clear supernatant fluid ; this is removed, and the bacterial mass is spread out in sterile Petri dishes, and dried for several days *in vacuo* over concentrated sulphuric acid. The dry scaly material is reduced to powder by grinding in Griffith's tubes, and is stored in sterile weighing bottles over calcium chloride in the cold.

The fractionation of the acetone-treated, dried bacterial bodies was carried out as follows :

To each of ten 500 c.c. conical flasks, each containing 200 c.c. sterile distilled water, were added 1 gm. of the dried bacterial bodies and 0·05 gm. trypsin (B.D.H., containing also erepsin). To each flask were also added 1·6 c.c. N/10 NaOH, giving a pH of 8·5, and 10 c.c. toluene. Each flask, fitted with a rubber cap, was incubated at 37° C. and frequently shaken. After 2½ days' incubation a further 0·05 gm. trypsin was added to each flask and the incubation continued for a total period of 5 days. At the end of the incubation period (final pH 8·5) the bulked material was evaporated *in vacuo* with a bath temperature not exceeding 40° C. until the whole of the toluene had been removed. The mixture was centrifuged and the somewhat opalescent supernatant separated. The residue was carefully washed with distilled water,

centrifuged, and the washings added to the main bulk. The insoluble residue was washed with alcohol and ether and dried ; its weight was 1·46 gm.

The mixed supernatant and washings were evaporated *in vacuo* to about 100 c.c. and transferred to a weighed 750 c.c. conical flask, which was then re-weighed. The weight of the solution was 137 gm. To every 32 gm. of the solution was now added 68 gm. of filtered absolute ethyl alcohol. The precipitate was allowed to settle overnight, was centrifuged, washed with 68 per cent. (by weight) alcohol, followed by absolute alcohol and ether, and dried. This constitutes Fraction F68, the weight of which in two separate experiments was 2·85 gm. and 2·30 gm.

2·50 gm. of Fraction F68 was suspended in 121·2 c.c. sterile distilled water and agitated for two hours at 37° C. The mixture was centrifuged, giving an opalescent supernatant which was separated, and a gelatinous residue. The gelatinous residue was extracted with a further 121 c.c. of sterile distilled water for half an hour at 37° C. with agitation and was centrifuged. The supernatant washings were combined with the supernatant solution referred to above, and the residue was washed with absolute alcohol and ether and dried, giving Fraction F68/U, weight 0·10 gm.

To the combined supernatant solution and supernatant washings was added an equal weight of filtered absolute ethyl alcohol, giving a precipitate which was spun off, washed with alcohol and ether and dried, and constitutes Fraction F68/50, weight 0·50 gm.

To the weighed supernatant from F68/50 was added sufficient filtered absolute ethyl alcohol to bring the concentration of alcohol to 60 per cent. by weight. The resultant precipitate was spun off, washed with alcohol and ether and dried, and constitutes Fraction F68/60, weight 0·26 gm.

To the weighed supernatant from F68/60 was added alcohol to bring the concentration of alcohol to 68 per cent. by weight. The resultant precipitate was spun off, washed with alcohol and ether and dried, giving Fraction F68/68, weight 0·31 gm.

The supernatant from F68/68 was evaporated *in vacuo* to about 20 c.c., and filtered absolute ethyl alcohol was added until no further precipitate was obtained. A sticky mass separated from which the supernatant was poured off, and the residue was ground with absolute alcohol followed by ether and dried, giving Fraction F68/95, weight 0·19 gm.

A second refractionation of F68 was carried out by Mr. A. R. Martin on identical lines, starting with 2·0 gm. of F68 treated with a proportionately equal amount of water. The weights of the various fractions that he obtained were as follows : F68/U 0·04 gm., F68/50 0·56 gm., F68/60 0·30 gm., F68/68 0·11 gm., F68/95 0·28 gm.

The fractions obtained in the first series of reprecipitations were tested in the usual manner, together with the standard preparation and the original F68. The results are set out in Table I. All fractions except F68/95 were tested simultaneously, with a single control group. The separate control group tested with F68/95 is included in Table I. In the case of F68/50 only 27 mice were available for test owing to deaths during the immunization period. Here, and in all subsequent tables, the standard errors of the percentage survivorships and mean times to death are included.

TABLE I.—*Showing the Effect of Immunizing Mice with Two Doses of* 0·017 *mgm. of Different Fractions of* Bact. aertrycke.

Fraction tested.	Number of mice.	Per cent. survivors.	Mean time to death.
Standard	30	10·00 ± 5·477	13·52 ± 0·848
F68	30	13·33 ± 6·206	15·58 ± 0·975
F68/U	30	23·33 ± 7·722	12·39 ± 0·920
F68/50	27	33·33 ± 9·072	16·00 ± 1·042
F68/60	30	26·67 ± 8·074	19·23 ± 0·902
F68/68	30	46·67 ± 9·108	15·25 ± 1·446
Control	30	0·00	5·17 ± 0·296
F68/95	30	[10·00 ± 5·477]	[7·41 ± 0·694]
Control (F68/95)	30	[6·67 ± 4·554]	[5·93 ± 0·924]

It will be noted that Fractions F68/U, F68/50, F68/60 and F68/68 appear to be rather more effective than F68 or than the standard preparation, as judged by the percentage of survivors, and that F68/68 gives, in this series of tests, the most satisfactory results. F68/95, which should be compared with its own control, produces no significant increase in resistance.

In an attempt to differentiate further between the relative immunizing values of the different fractions, this test was repeated, using two immunizing doses of 0·00017 mgm., an amount that, in earlier tests, had been found to be almost without effect in the case of the standard preparation. The results of this test are set out in Table II.

TABLE II.—*Showing the Effect of Immunizing Mice with Two Doses of* 0·00017 *mgm. of Different Fractions of* Bact. aertrycke.

Fraction tested.	Number of mice.	Per cent. survivors.	Mean time to death.
Standard	30	3·33 ± 3·277	9·10 ± 1·301
F68	30	13·33 ± 6·206	9·81 ± 1·033
F68/U	30	33·33 ± 8·606	10·55 ± 0·779
F68/50	30	16·67 ± 6·805	10·52 ± 0·960
F68/60	30	13·33 ± 6·206	10·62 ± 1·145
F68/68	30	13·33 ± 6·206	7·12 ± 0·908
F68/95	30	0·00	6·50 ± 0·686
Control	30	6·67 ± 5·554	5·93 ± 0·924

In this minute dose the immunizing effect of most of the fractions is minimal. Taking as our test the mean time to death of those mice that die within the period of observation, the difference between the test and control groups exceeds three times its standard error only in the case of Fractions F68/U, F68/50 and F68/60, though there is a suggestion of some increase in the resistance of the mice immunized with the standard preparation and with F68. Fraction F68/95 is quite inactive, and Fraction F68/68 has no significant effect. Taking the percentage of survivors as our test, only the mice immunized with Fraction F68/U show any significant difference as compared with the control group.

It will be noted that, with a hundredfold decrease in dose, the order of apparent activity of the fractions is reversed. With a dose of 0·017 mgm. F68/68 gives the maximal effect. With a dose of 0·00017 mgm. only F68/U induces any appreciable degree of immunity.

It would, we think, be unwise to stress any of these differences on the basis of the data at present available. Casper (1928) notes that the trivial increase in resistance which he records was induced by small but not by larger doses of his preparation, and in the parallel case of the pneumococcus there are several recorded observations suggesting that minute doses of an antigenically active polysaccharide may be more effective than larger ones (Schiemann and Casper, 1927; Wadsworth and Brown, 1931, 1933; Felton, 1932; Zozaya and Clark, 1933; Avery and Goebel, 1933).

It is possible that this factor of optimal dosage is responsible for the differences noted above, and if so it would seem logical to regard the fraction that produces a significant effect in the smallest dose as the most active. But a comparison of Tables I and II will show that in no case has a decrease in dose led to an increase in activity, and that in all cases except that of F68/U the decrease in activity with decrease in dose has been well marked. It is one of the difficulties of work of this kind that the solution of a single quantitative problem, such as the one here raised, demands the sacrifice of many hundreds of mice. For the moment we do not think it is justifiable to go beyond the conclusion that the immunologically active substance is present in each of the fractions F68/U, F68/50, F68/60 and F68/68.

The remainder of the immunological experiments outlined in this paper have been carried out with Fraction F68/68.

The Effect of Acid and Alkaline Hydrolysis on the Immunizing Potency of Fraction F68/68.

A solution of F68/68 in distilled water was made up containing 0·068 mgm. per c.c. Three portions of this solution of 10 c.c. each were treated as follows:

(*a*) 10 c.c. + 5 c.c. N/5 HCl heated in a boiling water bath for 30 minutes, cooled and exactly neutralized with 5 c.c. N/5 NaOH.

(*b*) 10 c.c. + 5 c.c. N/5 NaOH heated in a boiling water bath for 30 minutes, cooled and exactly neutralized with 5 c.c. N/5 HCl.

(*c*) 5 c.c. N/5 HCl exactly neutralized with 5 c.c. N/5 NaOH, and to the mixture 10 c.c. of the solution added.

0·5 c.c. of each of the above solutions (*a*), (*b*) and (*c*) thus contained the equivalent of 0·017 mgm. of F68/68.

The solutions so prepared, after standing overnight in the cold room at 0° C., were injected into mice, each mouse receiving the standard dose of 0·017 mgm. Fresh solutions were prepared and injected one week later. Another group of mice received two doses of 0·017 mgm. of the standard preparation. All the treated mice and 30 untreated controls were tested by the injection of 1000 living bacilli one week after the second immunizing injection.

The results are set out in Table III.

TABLE III.—*Showing the Effect of Acid and Alkaline Hydrolysis on Fraction F68/68.*

Fraction tested.	Number of mice.	Per cent. survivors.	Mean time to death.
Standard . . .	30	30·00 ± 8·367	13·62 ± 1·443
F68/68 unhydrolysed .	30	20·00 ± 7·303	11·79 ± 1·123
F68/68 acid-hydrolysed	30	0·00	5·60 ± 0·352
F68/68 alkaline-hydrolysed	30	10·00 ± 5·477	7·67 ± 0·746
Control . . .	30	0·00	7·67 ± 0·905

It will be seen that either acid or alkaline hydrolysis completely deprives F68/68 of its immunizing effect. The difference in percentage survivorship between the control mice and those immunized with the alkaline-hydrolysed preparation is insignificant, while the more delicate test of mean time to death reveals no difference between these groups. The mice immunized with the acid-hydrolysed preparation died slightly sooner than the controls. There is a suggestion that F68/68 loses some of its activity when allowed to stand in very dilute solution, since the preparation so treated was somewhat less effective than the standard suspension, while in previous trials the immunizing fractions showed a higher potency than the intact bacterial bodies; but this difference should not be stressed in the absence of further trials.

The Nature of the Immunity Induced.

It was of obvious importance to determine whether the immunity induced by the injection of the isolated fractions was of the same kind as that induced by the injection of intact bacterial bodies. Experiments were therefore made to test whether the injection of F68/68 gave rise to the formation of antibodies in mice or rabbits, and whether the active antibacterial immunity induced in mice could be passively transferred.

One hundred and twenty mice were given three injections of 0·017 mgm. of F68/68 at weekly intervals. Twelve days after the last injection 64 of the surviving 114 mice were bled to death under anæsthesia. The blood of 56 was pooled, that of the other 8 being collected separately. At the same time the blood of 50 normal mice was collected and pooled.

The sera were tested against an alcoholized suspension of *Bact. aertrycke*. Of the 8 immunized mice whose sera were tested separately, 3 showed somatic agglutination at a dilution of 1 : 10. The pooled immune sera showed agglutination at 1 : 20. The pooled normal sera showed no agglutination. These results are very similar to those we have obtained in many earlier experiments in which mice were immunized with unfractionated *Bact. aertrycke*.

On the following day 30 normal mice were injected intraperitoneally with 0·25 c.c. of the undiluted immune mouse serum, 30 with 0·25 c.c. of a 1 : 10 dilution of this serum, 30 with 0·25 c.c. undiluted normal mouse serum, and 30 with 0·25 c.c. of a 1 : 10 dilution of normal serum. One hour later these

mice, together with 30 untreated controls and 30 of the thrice-immunized mice of the group from which the immune serum was obtained, were injected with 10,000 living *Bact. aertrycke.* Ten times the standard infective dose was employed in this experiment to provide against the possibility that the normal mouse serum would have some protective effect. The results of this test are set out in Table IV.

TABLE IV.—*Showing the Effect of Passive Immunization with Immune Mouse Serum as Compared with the Effect of Active Immunization.*

Group.	Number of mice.	Per cent. survivors.	Mean time to death.
Active (F68/68) . .	30 .	13·33 ± 6·206 .	15·65 ± 1·112
Passive. Immune serum .	30 .	0·00 .	9·77 ± 0·478
,, ,, 1 : 10	30 .	3·33 ± 3·277 .	9·52 ± 0·724
Control. Normal serum .	30 .	0·00 .	3·70 ± 0·263
,, ,, 1 : 10	30 .	0·00 .	4·03 ± 0·213
,, Untreated . .	30 .	0·00 .	4·70 ± 0·372

It will be seen that the normal mouse serum had no protective effect. The immune serum had little if any effect in saving the life of the mice receiving it, but a definitely significant effect in prolonging the mean time to death. The differences are small, but so are the standard errors. During the first week after the test inoculation the differences in death-rate between the immunized and control groups were large. It seems possible that if the injection of serum had been repeated after infection more of the passively immunized mice might have survived.

Five rabbits, weighing from 1500 to 1650 gm., received intravenously 5 injections of F68/68, in doses of 0·1 mgm., 0·2 mgm., 0·2 mgm., 0·2 mgm., and 0·3 mgm. per kilo body-weight, given at weekly intervals. Before the first injection the serum of each rabbit was tested against an alcoholized suspension of *Bact. aertrycke* at a commencing dilution of 1 : 10. No agglutination was obtained. Six days after the fifth injection the rabbits were bled again and their sera tested against the same suspension. All gave well-marked somatic agglutination at titres varying from 1 : 160 to 1 : 640. The reactions of these rabbit antisera will be referred to again in a later section.

From these experiments we may conclude that Fraction F68/68, and inferentially the other immunizing fractions, induce an antibacterial immunity of the ordinary type associated with the production of antibodies acting on the surface somatic antigens of the virulent smooth form of *Bact. aertrycke.*

These immunizing fractions are, however, toxic—a point that is dealt with more fully in the two following papers—and it was clearly desirable to determine whether the immunity induced by their injection was antitoxic as well as anti-bacterial. For this purpose 20 mice that had received three immunizing injections of 0·017 mgm. of F68/68 were injected, together with 20 normal mice, with falling doses of this fraction, varying from 2·0 to 0·25 mgm. The mice were observed for a period of 96 hours. The results are shown in Table V.

TABLE V.—*Deaths among Immunized and Normal Mice after Injection of Falling Doses of F*68/68.

Dose.	Immunized.		Controls.	
	Number tested.	Number died.	Number tested.	Number died.
2·0 mgm.	5	2	5	5
1·0 ,,	5	1	5	5
0·5 ,,	5	1	5	4
0·25 ,,	5	0	5	2

It is clear that there is some degree, at least, of immunity to the toxic action of F68/68 as well as to infection with living bacilli. Observations on immunized rabbits are recorded in the following paper.

The Nature and Distribution of the Antigenic Material Present in the Various Fractions.

The fact that rabbits respond to the injection of F68/68, and, inferentially, of any of the other immunizing fractions, by the formation of specific antibodies, has enabled us to carry out a series of preliminary experiments on the nature and distribution of the corresponding antigens. The only serum so far available has been that prepared by the injection of F68/68 ; and since this part of our inquiry is as yet very incomplete we may summarize the significant results, leaving detailed protocols for a subsequent report.

It has been stated that the F68/68 antiserum gives typical somatic agglutination with an alcoholized suspension of *Bact. aertrycke*. It was clearly desirable to show that this depended on the presence of antibodies to the smooth somatic antigens differentiated by the well-known studies of White and of Kauffmann (see Lovell, 1932). For this purpose the antiserum was tested against a variety of Salmonella types, some sharing with *Bact. aertrycke* the antigens I and II (White), some containing only antigen II, and some containing neither of these antigens. Alcoholized suspensions of types containing antigens I and II (*Bact. aertrycke* and *Bact. paratyphosum B*) or II only (*Bact. abortus equi* and *Bact. abortus ovis*) showed well-marked somatic agglutination. The presence of antibodies corresponding to both antigens I and II in the F68/68 antiserum has been demonstrated by absorption tests. Thus, absorption of the antiserum with *Bact. paratyphosum B* (containing antigens I and II) removes the agglutinins for this organism and for *Bact. abortus equi* and *Bact. abortus ovis* ; but absorption with *Bact. abortus equi* or *Bact. abortus ovis* (containing only antigen II) completely removes the agglutinins for these two organisms, but causes only a slight reduction in the titre for *Bact. paratyphosum B*.

Tests on formolized suspensions of *Bact. aertrycke* and of other Salmonella types have shown, as would be expected, that flagellar antibodies are absent from the F68/68 antiserum.

These series of tests have, however, revealed the presence in this antiserum

of antibodies other than those acting on the somatic antigens I and II, and inferentially of corresponding antigens in Fraction F68/68. Earlier studies (Topley and Ayrton, 1924) have shown that broth cultures of Salmonella and allied organisms that have been incubated at 37° C. for 24 hours or more may be sensitive to agglutination with a variety of antisera developed against antigenically different types. This sensitiveness is increased by prolonged incubation of the cultures, and is sometimes, but not frequently, displayed by saline suspensions of growths on solid media. The conditions that tend to its development are those that allow the occurrence of some degree of bacterial autolysis. The phenomenon has been studied in greater detail by Happold (1928, 1929). For want of a better name the antibodies causing this type of agglutination were referred to as "x agglutinins". The bacterial antigens to which they correspond have not yet been identified, though Happold gives reasons for believing that they contain a protein component. Serum F68/68 has given clear indications of the presence of this x antibody. It agglutinates, to low titre, certain killed broth cultures of organisms that are unrelated to *Bact. aertrycke* in regard to their characteristic somatic antigens, such as *Bact. paratyphosum C* and *Bact. sanguinarium*, and control tests with a high titre anti-x serum have shown that the cultures so agglutinated are sensitive to x agglutinins.

In addition to this x antigen it would seem that Fraction F68/68 contains at least one other antigenic component. When the F68/68 antiserum is tested against alcoholized suspensions of various Salmonella types, the results are usually negative in the case of suspensions that contain neither antigen I nor II, and are insensitive to x agglutinins. Thus no somatic agglutination occurs with suspensions of *Bact. paratyphosum A* or *Bact. paratyphosum C*. But there are certain exceptions. An alcoholized suspension of *Bact. typhosum*, insensitive to x agglutinins, has given well-marked agglutination, indistinguishable from the characteristic somatic type, and a similar suspension of *Bact. enteritidis* has given agglutination to a lower titre. This does not appear to be due to the presence of an antibody corresponding to White's antigen III, the characteristic somatic antigen of these types, since somatic agglutination does not occur with *Bact. dublin* and *Bact. sanguinarium*, both of which share the same antigen. It seems more probable that it is due to the presence in F68/68 of a minor antigenic component shared by *Bact. aertrycke*, *Bact. typhosum* and *Bact. enteritidis*.

We may conclude that our immunizing and toxic fractions contain the antigens I and II in an immunologically active form, and traces at least of other antigenic components.

Preliminary tests with serum F68/68 and the corresponding fraction gave a well-marked precipitation reaction. This obviously afforded an opportunity to determine approximately the distribution of the precipitable antigen or antigens in the other immunizing or non-immunizing fractions.

Precipitation tests have been carried out using the optimal-proportions technique of Dean and Webb (1926). These tests have shown that the weights of the various dried fractions that give optimal flocculation with 0·1 c.c. of serum F68/68, when dissolved in 1 c.c. of saline and incubated at 55° C., are approximately as follows :

F68/U	0·2 –0·3 mgm.
F68/50	0·16–0·25 ,,
F68/60	0·16–0·25 ,,
F68/68	0·16–0·25 ,,

The non-immunizing fraction F68/95 gave no precipitation at any point over the range 0·0025–10 mgm.

We have not yet had available sufficient material to carry out titrations in which successive tubes differ but little in their antigen-content, so that we should regard the figures given above only as rough approximations. With this limitation they suggest that the various fractions over the range F68/U to F68/68 do not differ greatly in their content of the essential antigenic constituent.

These tests have, however, given a clear indication of some difference in the state of this antigen in the different fractions, since the optimal flocculation times have differed widely. Fraction F68/U gave almost immediate flocculation, so rapid that the optimal tube was by no means easy to determine. Fractions F68/50 and F68/60 showed first flocculation after about 47 minutes. Fraction F68/68 showed first flocculation after about 77 minutes. It is possible that these differences are related to differences in the state of aggregation of the antigenic material, since they show a general correspondence with the opalescence of the solutions. If this is so, the optimal ratio figures given above may be affected by the same factor. Alternatively, it is possible that there may be differences in chemical structure, perhaps in the amount of the antigenic compound that is present in the combined, as contrasted with the disrupted state ; or the differences in flocculation rate may be due to the presence in unequal amounts of some non-specific contaminant that hastens flocculation.

The Chemical Properties of the Various Fractions.

Portions of the different fractions were dried to constant weight *in vacuo* at 60° C., and micro-analyses were carried out on the dried material by Schoeller, Berlin. The results are given in Table VI, items 1–6. The figures indicate that probably none of the fractions is a chemical entity.

TABLE VI.—*Quantitative Analysis of the Different Fractions.*

Test No.	Test.	Fraction F68/U.	Fraction F68/50.	Fraction F68/60.	Fraction F68/68.	Fraction F68/95.
1	% Carbon	43·64	39·55	38·78	38·57	38·01
2	% Hydrogen	6·46	5·84	6·01	5·95	6·34
3	% Nitrogen	6·50	3·64	3·94	4·70	14·06
4	% Sulphur	0·99	0·69	0·83	1·03	0·52
5	% Phosphorus	1·87	3·40	2·96	2·24	3·52
6	% Non-combustible residue	..	16·32	13·68	10·74	12·76
7	% Reducing sugars on hydrolysis	28·80	31·20	35·70	37·80	8·90

Fraction F68 gave 23·1 per cent. reducing sugars on hydrolysis.

The estimation of the reducing sugars formed on hydrolysis given in item 7 was kindly carried out for us by Prof. Delafield. The method followed was to hydrolyse a weighed amount (8 mgm. to 34 mgm.) of each fraction at 100° C. for one hour with 1–2 c.c. N/1 HCl. The hydrolysis solution was then carefully neutralized with N/1 NaOH, and the reducing sugars estimated by the Hagedorn-Jensen method.

A series of qualitative chemical tests was carried out on 0·5 c.c. portions of a 0·5 per cent. solution of Fractions F68, F68/50, F68/60 and F68/95. The results are summarized in Table VII. Tests with sulphosalicylic acid and picric acid (Esbach's reagent) indicate the entire absence from all fractions examined of intact protein. The almost negligible precipitates obtained with phosphotungstic acid with Fractions F68/50 and F68/60 indicate the probable absence of partially degraded protein. On the other hand the positive results obtained with the biuret and ninhydrin tests appear to indicate the presence of peptide linkages, although in this connection it should be noted that the reddish-purple colour obtained with ninhydrin was quite different in shade from the usual blue colour given with amino-acids and peptides. The Millon reaction was carried out using the improved technique, in which a sulphuric acid solution of mercuric sulphate is added to the test solution, heated, cooled, a drop of 1 per cent. $NaNO_2$ solution added, and then warmed, a reddish colour on warming indicating the presence of phenolic groupings. The Millon reaction revealed the interesting fact that there is present in Fraction F68/95, and to a less extent in Fraction F68, some material, presumably nitrogenous, which is precipitable by acid mercuric sulphate. This material is entirely absent in Fractions F68/50 and F68/60.

The Molisch reaction was strongly positive with each fraction.

The exact form in which the relatively large amounts of sulphur is present in the different fractions has not yet been determined with certainty, although it is certainly not present as inorganic sulphate.

The phosphorus is present partly in a form precipitable by magnesia mixture and partly as phosphorus in organic combination. The evidence for this statement is the production by all fractions of a crystalline precipitate with excess of magnesia mixture. This crystalline precipitate was filtered off, the clear filtrate evaporated to dryness, oxidized with fuming nitric acid and tested with ammonium molybdate. The production of the typical yellow phosphomolybdate with all fractions indicates the presence of organic phosphorus.

Four c.c. portions of 0·5 per cent. solutions of each fraction were hydrolysed by the addition of sufficient concentrated HCl to give a N/1 HCl solution, which was then heated at 100° C. for one hour. On cooling, each of the fractions F68, F68/50 and F68/60, but not F68/95, deposited an amorphous precipitate which was partly soluble in ether. The ether solution in each of the three cases mentioned left on evaporation a partially crystalline fatty residue. The filtered hydrolysis solutions from Fractions F68, F68/50 and F68/60 all gave a strong reduction with Fehling's solution and a crystalline osazone on heating with phenylhydrazine in acetic acid solution. On the other hand, Fraction F68/95 gave a negligible reduction with Fehling and no osazone.

The chemical facts presented indicate quite clearly the presence in the biologically active fractions tested of a carbohydrate nucleus. Further, there

TABLE VII.—*Qualitative Chemical Examination of Solutions of the Different Fractions.*

Test No.	Test.	Fraction F68.	Fraction F68/50.	Fraction F68/60.	Fraction F68/95.
1	Sulphosalicylic acid	Negative	Negative	Negative	Negative.
2	Picric acid (Esbach's reagent)	,,	,,	,,	,,
3	Phosphotungstic acid	Slight precipitate	Very slight precipitate	Very slight precipitate	Heavy amorphous precipitate.
4	Biuret	Definitely positive	Slight but positive	Slight but positive	Definitely positive.
5	Ninhydrin	Reddish-purple colour	Pale reddish-purple colour.	Pale reddish-purple colour.	Strong reddish-purple colour.
6	Millon	Slight white gelatinous precipitate on addition of reagent. Red precipitate on warming	No precipitate on addition of reagent. Pink colour on warming; red precipitate overnight	No precipitate on addition of reagent. Pink colour on warming; red precipitate overnight	Heavy white gelatinous precipitate on addition of reagent. Red precipitate on warming.
7	Molisch	Strong positive	Strong positive	Strong positive	Strong positive.
8	$BaCl_2$ with or without HCl	Negative	Negative	Negative	Negative.
9	S–H (after reduction with KCN) by nitroprusside reaction	Slight but definitely positive	Negative	Negative	Definitely positive.
10	Magnesia mixture	Crystalline precipitate	Crystalline precipitate	Crystalline precipitate	Crystalline precipitate.
11	Phosphorus not precipitable with magnesia mixture	Positive	Positive	Positive	Positive.

can be no question of the presence in these fractions of any intact protein. On the other hand there are definitely present nitrogen compounds of a peptide nature, but whether these are an integral part of the biologically active molecule we are not at present in a position to state. The relatively high percentage of sulphur, particularly when considered in relation to the relatively low percentage of nitrogen, appears to indicate, although not to prove, the presence of a sulphur-containing grouping in the biologically active molecule. The presence of organic phosphorus coupled with the presence of material of a fatty nature in the biologically active fractions is presumptive evidence of a phosphatide grouping as part of the molecule.

It is obvious that any opinions as to the chemical make-up of the immunologically active molecule would at this stage be premature. We hope, however, by continued fractionation ultimately to obtain chemical entities and then to examine the chemical make-up of these entities.

DISCUSSION.

In discussing the significance of the results that we have so far obtained, it is necessary to bring them into relation with the recent observations to which we have already referred.

The studies of Avery and Goebel (1933) have shown that the presence of an acetyl grouping in the natural Type I pneumococcal polysaccharide confers on it the power of inducing active immunity in mice—a power that is lost in the de-acetylated product resulting from the methods of isolation that had been previously employed by them. The acetylated polysaccharide, however, differs in its immunological activity from our active fractions in that it fails to stimulate antibody-production *in vivo*. So far as our experiments have gone, they do not suggest that the activity of our fractions is likely to be dependent upon the presence of an acetyl or similar grouping in the antigenic molecule.

There can, we think, be little doubt that the fraction isolated from *Bact. aertrycke* by Boivin and his colleagues (1933, 1934) by extraction with trichloracetic acid is either identical with or closely related to the active immunizing fraction that we have isolated by another method. The immunological reactions of Boivin and Mesrobeanu's product have not yet been fully described, but such data as are recorded are in entire agreement with the results that we have obtained. Thus, the trichloracetic acid extract, after further purification, is toxic for mice and for rabbits, and when injected into rabbits it induces some degree of antitoxic immunity associated with the production of specific agglutinins and precipitins. There is no record of any experiments on the production of immunity to infection with living bacilli, but the stimulation of agglutinin formation suggests that such an immunity would be induced.

By boiling a solution of their toxic and immunizing fraction with N/5 acetic acid Boivin and his colleagues have separated a soluble and an insoluble fraction. The former is non-toxic and fails to stimulate antibody production, but flocculates with an anti-*aertrycke* serum. They regard it as the polysaccharide hapten component of the complex antigenic compound. The acetic acid insoluble fraction, dissolved in cold dilute sodium hydroxide, is

highly toxic for mice, but gives no precipitate with an anti-*aertrycke* serum. They regard it as the component that confers toxicity and immunizing potency on the complex antigenic compound and, for reasons that are referred to in the following paragraphs, believe it to be a phosphatide.

Our chemical findings confirm and extend those of Boivin and his colleagues. These authors report a yield of 40 per cent. of reducing sugars expressed as glucose, while our figures are 28·8 per cent., 31·2 per cent., 35·7 per cent. and 37·8 per cent. for the four immunizing fractions F68/U, F68/50, F68/60 and F68/68 respectively. Further, they report the production after boiling with N/5 acetic acid of a colourless precipitate, partly soluble in ether, which clearly corresponds to the ether-soluble substance formed on hydrolysis with N/1 HCl of our active material. We agree also in each recording a positive Millon reaction which, of course, does not necessarily prove the presence of tyrosine in the molecule, since many phenolic bodies give a similar reaction. Since Boivin and his colleagues give no chemical analyses for their active material no comparison with ours can be made. They do, however, record the presence of organic phosphorus and organic nitrogen, with which, of course, we agree, but have not reported the presence of sulphur. The presence of sulphur in an organic form in our active fractions, in amounts averaging about 1 per cent., appears to us to be of considerable significance. It can scarcely be that this sulphur is present solely as a constituent of an accompanying impurity, since the respective percentages of nitrogen and sulphur in our active immunizing fractions are 6·50, 3·64, 3·94 and 4·70 per cent. nitrogen, and 0·99, 0·69, 0·83 and 1·03 sulphur in fractions F68/U, F68/50, F68/60 and F68/68 respectively. This is in marked contrast to the relative nitrogen and sulphur content of the biologically inactive Fraction F68/95 (14·06 per cent. N and 0·52 per cent. S) and of proteins, which contain from 15 to 19 per cent. nitrogen and from 0·4 to 2·5 per cent. sulphur (Plimmer).

Boivin and his colleagues regard the ether-soluble fraction of the acetic acid hydrolysed active material as a phosphatide " because of its general properties and content of C, N and P ". Such evidence as we have presented in this report is not incompatible with this view, but until further evidence is available it must be regarded as an unproven hypothesis.

SUMMARY.

In summary, we think it has been demonstrated that fractions can be isolated from *Bact. aertrycke*, and inferentially from other organisms, that contain the specific somatic polysaccharides in an antigenically active form in the absence of any intact protein. Such fractions are toxic, and are efficient immunizing agents, inducing an active antibacterial and antitoxic immunity associated with the production of specific precipitating and agglutinating antibodies. As the following papers will show, the distribution of toxicity among the various fractions runs closely parallel to the distribution of immunizing potency. Whether this indicates that the toxic and immunizing substances are identical, or whether we are dealing with two or more different substances that behave in the same way to the reagents we have employed, or with an association resulting from adsorption, are problems that await further solution.

It need hardly be added that there would be no justification for the assumption that the toxic and immunizing substances present in our fractions represent the total toxic and immunizing potentialities of the bacterial cells.

The fractions with which we have been concerned are certainly not chemically pure substances, and we do not think that the evidence at present available, although indicative of the presence of a phosphatide as well as a polysaccharide, allows any final conclusion to be reached as to the exact structure of the complete antigenic molecule of which the polysaccharide forms a part.

The problem of chemical purification and identification suggested by the data set out above is not a simple one, and it seems likely that much more work will be required before it is solved. We hope to continue this investigation, and to extend its range to other bacteria, in collaboration with Prof. R. Robinson of Oxford and Prof. W. N. Haworth of Birmingham. There would seem to be a reasonable hope that we may be able to replace some of our crude immunizing extracts and suspensions by known chemical entities, and in so doing gain an added precision in our experimental work, and perhaps in practical prophylaxis.

In conclusion we should wish to express our thanks to Mrs. Joyce Wilson for her help in the preparation of our bacterial growths and in many other ways, to Dr. J. H. Birkinshaw, Dr. A. E. Oxford and Dr. M. Stacey, who have helped us on various occasions in the preparation or handling of our fractions, and to Mr. W. K. Anslow for much technical assistance.

REFERENCES.

ARKWRIGHT, J. A.—(1927) *J. Path. Bact.*, **30**, 345.
AVERY, O. T., AND GOEBEL, W. F.—(1931) *J. Exp. Med.*, **54**, 437.—(1933) *Ibid.*, **58**, 731.
AVERY, O. T., AND MORGAN, H. J.—(1925) *Ibid.*, **42**, 347.
AVERY, O. T., AND NEILL, J. M.—(1925) *Ibid.*, **42**, 355.
BOIVIN, A., AND MESROBEANU, L.—(1933) *C. R. Soc. Biol.*, Paris, **112**, 76.—(1934) *Ibid.*, **115**, 304, 309.
BOIVIN, A., MESROBEANU, I., AND MESROBEANU, L.—(1933) *Ibid.*, **113**, 490; **114**, 307; (1934) *Ibid.*, **115**, 306.
CASPER, W.—(1928) *Z. Hyg. InfektKr.*, **109**, 170.
COMBIESCO, D., STAMATESCO, S., AND SORU, E.—(1930) *Arch. roum. Path. exp. Microbiol.*, **3**, 189.
DEAN, H. R., AND WEBB, R. A.—(1926) *J. Path. Bact.*, **29**, 473.
DOUGLAS, S. R.—(1923) *Brit. J. Exp. Path.*, **2**, 175.
Idem AND FLEMING, A.—(1923) *Ibid.*, **2**, 131.
FELIX, A.—(1924) *J. Immunol.*, **9**, 115.
FELTON, L. D.—(1932) *Ibid.*, **23**, 405.
FURTH, J., AND LANDSTEINER, K.—(1928) *J. Exp. Med.*, **47**, 171.—(1929) *Ibid.*, **49**, 727.
GREENWOOD, M., TOPLEY, W. W. C., AND WILSON, J.—(1931) *J. Hyg.*, Camb., **31**, 257.
GRINNELL, F. B.—(1932) *J. Exp. Med.*, **56**, 907.
HAPPOLD, F. C.—(1928) *J. Path. Bact.*, **31**, 237.—(1929) *Brit. J. Exp. Path.*, **10**, 263.
HEIDELBERGER, M., AND AVERY, O. T.—(1923) *J. Exp. Med.*, **38**, 73.
HENDERSON, D. W.—(1932) *Brit. J. Exp. Path.*, **13**, 421.

IBRAHIM, H. M., AND SCHUTZE, H.—(1928) *Ibid.*, **9**, 353.
LOVELL, R.—(1932) *Bull. Hyg.*, **7**, 405.
MEYER, K.—(1931) *Z. ImmunForsch.*, **71**, 331.
PERLZWEIG, W. A., AND KEEFER, C. S.—(1925) *J. Exp. Med.*, **42**, 747.
PERLZWEIG, W. A., AND STEFFEN, G. I.—(1923) *Ibid.*, **38**, 163.
ROBERTSON, M., AND FELIX, A.—(1930) *Brit. J. Exp. Path.*, **11**, 14.
SCHIEMANN, O., AND CASPER, W.—(1927) *Z. Hyg. InfektKr.*, **108**, 220.
SCHIEMANN, O., LOEWENTHAL, H., AND HACKENTHAL, H.—(1931) *Ibid.*, **112**, 315.
TOPLEY, W. W. C., AND AYRTON, J.—(1924) *J. Hyg.*, Camb., **23**, 198.
WADSWORTH, A., AND BROWN, R.—(1931) *J. Immunol.*, **21**, 245.—(1933) *Ibid.*, **24**, 349.
WHITE, P. B.—(1929) *J. Path. Bact.*, **32**, 85.—(1931) *Ibid.*, **34**, 325.
ZINSSER, H., AND PARKER, J. T.—(1923) *J. Exp. Med.*, **37**, 275.
ZOZAYA, J., AND CLARK, J.—(1933) *Ibid.*, **57**, 21.

BLOOD-SUGAR CHANGES AND TOXIC EFFECTS PRODUCED IN RABBITS BY CERTAIN FRACTIONS DERIVED FROM *BACT. AERTRYCKE.*

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PREVIOUS work (Delafield, 1931, 1932) has shown that the intravenous injection into rabbits of the dead cells of *Bact. aertrycke* and of a considerable number of other Gram-negative organisms is followed by an early hyperglycæmia and toxic effects. Other workers (Menten and Manning, 1925; Zeckwer and Goodell, 1925) had made similar observations on a small number of different organisms. The preparation from the bodies of *Bact. aertrycke* by tryptic digestion followed by alcohol precipitation of the fractions described in the preceding paper, in regard to some of their chemical characters and their ability to induce antibacterial and antitoxic immunity, has provided an opportunity for an attempt to correlate the power to produce immunity with the capacity to produce hyperglycæmia and toxic effects in rabbits.

Those fractions which have antigenic and immunizing power are found to be the same as those which are active in the sense of producing hyperglycæmia and toxic effects in rabbits; they all contain polysaccharide components and no unaltered protein.

A number of workers have obtained fractions from bacterial bodies or from liquid cultures which are toxic in minute doses. Ecker and Rimington (1928) obtained from *Bact. aertrycke* grown on a liquid synthetic medium a fraction which was toxic, but not fatal, to the two rabbits tested in a dose of 0·07 mgm. but no determination of blood-sugar was made. Their method was to precipitate the concentrated dialysed culture with lead acetate. The lead precipitate

was then triturated with solid ammonium sulphate to remove the lead as sulphate and precipitate any protein present. On filtration the active substance was found in the filtrate which was purified by dialysis ; it remained in the dialysis sac. The substance gave a negative biuret reaction but a strong Molisch reaction, and contained about 0·4 per cent. of nitrogen. Menten and King (1930) obtained from a peptone broth culture of *Bact. aertrycke* a powder which, in a dose of 0·01 mgm., evoked a hyperglycæmic response in rabbits. The method was to precipitate with acetic acid and dissolve the precipitate in alkali, re-precipitate with acetic acid and again dissolve, and after a third such treatment to precipitate with 95 per cent. alcohol. The product contained about 11 per cent. of nitrogen, gave negative tests for sulphur and phosphorus, and reduced Benedict's solution after acid hydrolysis. A fraction obtained by precipitation of the liquid culture with ammonium sulphate contained phosphorus and sulphur, but did not produce hyperglycæmia. Boivin and collaborators (1933) treated the bodies of *Bact. aertrycke* with trichloracetic acid, centrifuged off the precipitate and dialysed the opalescent supernatant liquid. The active substance, which was a complex polysaccharide containing nitrogen and phosphorus, did not dialyse. Its effect on the blood-sugar was not examined, but it was lethal for rabbits in an intraperitoneal dose of 1·0 mgm. ; in smaller doses it caused the animal to develop resistance to lethal doses, and also evoked the production of agglutinins and precipitins.

EXPERIMENTAL.

Each fraction was tested on at least two rabbits, in the first instance by the intravenous injection of a uniform dose of 2·0 mgm. per kg. of body-weight. The substance was prepared for injection by solution or suspension in sterile saline in a concentration of 1·0 mgm. per c.c. Rabbits of about 2 kg. weight were used in most instances.

Blood-sugar was determined in duplicate by the Hagedorn and Jensen (1923) technique before injection and at 2 and 24 hours thereafter. Weight and temperature records were kept.

The Fractions Tested.

The method of preparation of these fractions is detailed in the preceding paper. The nomenclature there used to indicate the fractions will be used in this paper.

The Effect of the Alcohol Fractions in Producing Hyperglycæmia and Toxic Results.

In Table I are recorded the blood-sugar and weight changes following the injection of the different alcohol fractions, in comparison with the Standard preparation (acetone-treated bodies).

In addition to the Standard preparation the following fractions were active : (1) F68, (2) F68/U, (3) F68/50, (4) F68/60, (5) F68/68.

Entirely inactive in producing any blood-sugar changes or significant loss of weight was the F68/95 fraction, although the injected animals became apathetic and their circulation was depressed.

Fractions which were similarly active but are not recorded in Table I are : (1) bacterial bodies treated successively with acetone, ethyl ether, petroleum ether, chloroform and methyl alcohol ; (2) the residue left undissolved after digestion with trypsin.

TABLE I.—*The Effect of the Alcohol Fractions and of the Standard Preparation (Acetone Extracted Bodies) on Blood-sugar and Weight of Rabbits.*

Fraction.		Dose per kg. body-weight in mgm.	Blood-sugar in mgm. %. Hours. 0.	2.	24.	Percentage weight loss in two days.
Standard	(3)	2·0	132	298	87	17
F68	(2)	2·0	116	198	101	8
F68/U	(2)	2·0	107	182	82	16
	(2)	0·4	125	240	98	17
F68/50	(2)	2·0	116	204	87	16
	(2)	0·4	123	317	72	15
F68/60	(2)	2·0	116	380	87	11
	(2)	0·4	107	201	96	12
F68/68	(2)	2·0	124	333	80	17
	(2)	0·4	110	187	100	8
F68/95	(2)	2·0	116	118	108	..
	(2)	4·0	112	113	106	5

The number of animals tested is given in brackets. The figures are the mean of these test results.

The injection of doses of 0·4 or 2·0 mgm. per kg. body-weight of the active fractions is followed by obvious illness, which may be dramatic in the rapidity of its onset. Usually within fifteen minutes, but sometimes only after about an hour, the animal is subdued and breathing rapidly. Coarse tremors are common, as also is severe diarrhœa. Some of the animals have become completely prostrate and occasionally there is great weakness of the hind legs. At the end of two hours the animal is usually very limp, the temperature falls in severe cases, the peripheral circulation is always definitely depressed, usually to such an extent that cardio-puncture is necessary to obtain blood for analysis.

The blood-sugar rises by the end of two hours to a definite hyperglycæmic level, sometimes to a figure three or four times the initial one. In only one instance was there a negligible rise in blood-sugar, a significant level being taken as one upwards of 150 mgm. per 100 c.c. of blood. After 24 hours the blood-sugar has again fallen, and usually to a level lower than that of zero hour. This fall in blood-sugar is invariable, but a striking hypoglycæmia of about 60 mgm. per cent. or below has been noted only on few occasions. Previous work has shown (Delafield, 1931, 1932) that when death results

following the injection of dead *Bact. aertrycke*, or of certain other Gram-negative organisms, the blood-sugar shortly before death is always low and sometimes falls to convulsive level.

In the animals which survive the acute stage of illness usually passes off in about 4 hours, but the weight continues to fall for about 4 days during which time little food is eaten. As regards the lethal effect, the animals which have died have done so within the period of 2 to 48 hours after injection, but survival time cannot, in these experiments, be used as an index of toxicity owing to the fact that the cardio-puncture, which is often necessary, frequently causes a hæmopericardium in seriously affected animals, possibly due to a lowered coagulability of the blood.

It has not as yet been possible accurately to compare the different fractions as regards their toxic and hyperglycæmia-producing powers by the use of an extended range of dose or larger numbers of animals of the same strain.

Post-mortem Appearances.

No histological examination has as yet been undertaken, but a very striking naked-eye appearance is the almost invariable hæmorrhagic congestion of the thyroid. The mesenteric vessels are much injected, and there may be reddening of the pancreas and of the adrenals. Definite hæmorrhages may sometimes be seen in the walls of the intestines and occasionally in the adrenals and kidneys.

Inactive Fractions.

The effect of the inactive F68/95 is recorded in Table I, but it is perhaps significant that this fraction, though without effect on blood-sugar, did produce some depression of peripheral circulation and the injected animals became somewhat apathetic. The results of the other inactive fractions are recorded in Table II. These inert fractions are : (1) F95, the fraction precipitated from the tryptic digest by excess alcohol after the separation of the F68 precipitate : (2) the fraction of the digest soluble in alcohol ; (3) the fraction dissolved by 50 per cent. acetone-water from the bacterial bodies ; (4) the fraction dissolved by acetone from the bacterial bodies.

The Effect of Alkali and Acid Hydrolysis on the Activity of the F68/68 Fraction.

Weighed quantities of about 15 mgm. of the fraction were mixed with 2 c.c. of water and then heated in a boiling water bath for 30 minutes, one with the addition of 5 c.c. of N/5 NaOH and another with 5 c.c. of N/5 HCl. They were cooled and then neutralized to phenolphthalein with N/5 acid or alkali. A third quantity of the substance was dissolved in 10 c.c. of a mixture of exactly equal parts of N/5 alkali and acid, and titrated to the pink of phenolphthalein. Dilution of the three solutions to the required strength for injection was done with sterile saline. Injections into three groups of two animals each were then made with a dose equivalent to 2·0 mgm. of the fraction F68/68 per kg. body-weight.

There was no significant blood-sugar response in any of the animals injected

TABLE II.—*The Effect of the Other Inactive Fractions on Blood-sugar and Weight of Rabbits.*

Fraction.		Dose per kg. body-weight in mgm.	Blood-sugar in mgm. %. Hours. 0.	2.	24.	Percentage weight loss in two days.
1 F95	(2)	2·0	108	125	118	..
	(2)	4·0	120	120	119	0
2 Alcohol-soluble fraction	(2)	2·0	107	110	128	0
3 Acetone-water-soluble fraction	(2)	2·0	110	117	110	3
4 Acetone-soluble fraction	(2)	2·0	101	104	111	0

The number of animals tested is given in brackets. The figures are the mean of these test results. Fractions 1 and 2 were derived from a tryptic digest; fractions 3 and 4 from the supernatants obtained in the acetone treatment of the original bacterial suspension.

with the hydrolysed fractions. The rabbits given the alkali hydrolysed fraction did not appear at all ill and the weight loss was slight. Those that received the acid hydrolysed substance lost a considerable amount of weight, though less than the controls, and appeared not to be completely unaffected, as estimated by clinical observation of the circulation and the appearance of the animals.

Table III gives the numerical results.

TABLE III.—*The Effect of Unhydrolysed, Alkali-Hydrolysed and Acid-Hydrolysed F68/68 Alcohol Precipitate on Blood-Sugar and Weight of Rabbits.*

Fraction.	Dose per kg. body-weight in mgm.	Blood-sugar in mgm. %. Hours. 0.	2.	24.	Percentage weight loss in two days.
Unhydrolysed	2·0	121	226	85	15
,,	2·0	114	177	99	14
Alkali-hydrolysed	2·0	121	133	127	1
,,	2·0	139	121	110	6
Acid-hydrolysed	2·0	114	135	92	9
,,	2·0	167	122	96	12

The Production of Immunity to the Blood-sugar Response.

Three of the animals referred to in the preceding paper as having developed agglutinins and precipitins after five weekly injections of 0·1 to 0·3 mgm. per kg. body-weight of the fraction F68/68 were compared with 3 normal animals in their response to a 2·0 mgm. per kg. body-weight dose of the same fraction.

Table IV indicates that there is a significant difference between the two groups, only one of the immunized animals showing any significant blood-sugar rise, whereas all the normals gave the typical response. The experiment was repeated after an 8-day interval with the same immunized animals and 3 new normals. The total F68 fraction was used on this occasion in a dose of 4·0 mgm. per kg. body-weight. A similar immunity to the blood-sugar response was demonstrated in the immunized animals, only one of which, and the same one as in the previous experiment, showing any significant rise.

Clinically there was no marked difference between the two groups as regards appearance after injection, enfeeblement of the peripheral circulation and weight loss, but one of the normals died overnight in the second experiment.

TABLE IV.—*Blood-Sugar Response to Active Fractions in "Immune" and Normal Animals.*

Rabbit.		Dose per kg. body-weight in mgm.	Blood-sugar in mgm. %. Hours. 0.	2.	24.	Percentage weight loss in two days.
Experiment 1: Fraction used F68/68.						
"Immune"	473	2·0	111	107	91	8
,,	465	2·0	121	170	101	12
,,	499	2·0	94	122	102	9
Normal	550	2·0	112	180	85	10
,,	553	2·0	110	256	105	5
,,	547	2·0	108	347	110	7
Experiment 2: Fraction used F68.						
"Immune"	473	4·0	99	125	71	13
,,	465	4·0	108	170	101	11
,,	499	4·0	100	129	96	11
Normal	546	4·0	122	370	83	16
,,	540	4·0	101	211	83	17
,,	551	4·0	113	350	D.	..

DISCUSSION.

Although there is close parallelism between the antigenic and immunizing power of the fractions and their toxic and hyperglycæmia producing capacity, this does not of necessity indicate that the chemical substances producing these effects are one and the same. It is possible that there is only one substance responsible for both effects, but, on the other hand, there may be two or more substances linked or merely associated in the same alcohol fractions, and present in the different fractions in varying proportions or in different states of aggregation. In the recent work of Boivin and collaborators (1933),

previously quoted, on a "polysaccharide complex" obtained from *Bact. aertrycke* after removal of proteins by trichloracetic acid, it is suggested that there are two substances linked together—the one a polysaccharide substance responsible for the antigenic and immunizing reactions, the other a non-polysaccharide substance, perhaps a phosphatide, obtained as a precipitate by heating the total "complex" with acetic acid, which has toxic effects, but is not reactive with precipitating serum. The "polysaccharide complex" has many features in common with our active alcohol fractions, but their identity is not yet established, nor is it stated whether or not the former has any hyperglycæmic effect. Other problems arising out of this work on alcohol fractions which suggest themselves are whether there is only one toxic fraction in the bacterial bodies, and also whether the toxic effect is wholly due to the substance that brings about so profound an alteration in the carbohydrate metabolism. The bacterial bodies appear to be no less toxic than the active fractions in the doses employed and, as the latter represent only a proportion of the mass of the intact cells, it is a possibility that there are other toxic substances in the cells that are lost or inactivated during fractionation. Again, the effect of the acid-hydrolysed material in producing some general toxic effects but no hyperglycæmia may point to the existence in the unhydrolysed fraction of principles which are toxic but not hyperglycæmic. In contrast with these toxic and hyperglycæmic reactions produced by polysaccharide fractions from *Bact. aertrycke*, it is of interest that the polysaccharide substance derived from the pneumococcus is nowhere recorded as having any toxic effects on rabbits, although it may produce purpura in mice when administered in large doses, as stated by Schiemann and collaborators (1931) and Wadsworth and Brown (1933). Indeed, Avery and Goebel (1933), in their recent work on the acetyl polysaccharide of the pneumococcus, record the injection of 17 mgm. into a rabbit with survival of the animal. This difference between the polysaccharides of the two organisms corresponds with the differences in the results obtained when the dead bacterial cells are injected into rabbits. Of 12 Gram-negative organisms tested (Delafield, 1932), 8, including *Bact. aertrycke*, produced illness and hyperglycæmia, whereas *Str. pneumoniæ* and 10 other Gram-positive organisms did not produce, in identical opacity doses, either of these effects.

SUMMARY.

1. Fractions, containing polysaccharide components but no unaltered protein, derived from the bodies of *Bact. aertrycke* by tryptic digestion followed by alcohol precipitation, are active in producing hyperglycæmia and toxic effects in rabbits.

2. The toxicity of the various fractions tested runs closely parallel to their ability to induce antibacterial and antitoxic immunity in mice.

3. These active fractions are all precipitable within the range of 50 to 68 per cent. by weight of alcohol concentration.

4. The fraction obtained by excess alcohol after removal of the 68 per cent. fraction is inactive, as also is the alcohol-soluble material.

5. Acid or alkali hydrolysis of the F68/68 alcohol precipitate destroys the power to produce hyperglycæmia.

6. Immunity to the chemical response can be developed by a series of injections of the F68/68 alcohol fraction.

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REFERENCES.

AVERY, O. T., AND GOEBEL, W. F.—(1933) *J. Exp. Med.*, **58**, 731.
BOIVIN, A., MESROBEANU, I., AND MESROBEANU, L.—(1933) *C. R. Soc. Biol.*, Paris, **113**, 490.—(1933) *Ibid.*, **114**, 307.
DELAFIELD, M. E.—(1931) *J. Path. Bact.*, **34**, 177.—(1932) *Ibid.*, **35**, 53.
ECKER, E. E., AND RIMINGTON, C.—(1928) *J. Hyg.*, Camb., **27**, 44.
HAGEDORN, H. C., AND JENSEN, B. N.—(1923) *Biochem. Z.*, **135**, 46.
MENTEN, M. L., AND KING, C. A.—(1930) *J. Infect. Dis.*, **46**, 275.
Idem AND MANNING, H. M.—(1925) *Ibid.*, **37**, 400.
SCHIEMANN, O., LOEWENTHAL, H., AND HACKENTHAL, H.—(1931) *Z. Hyg. InfektKr.*, **112**, 315.
WADSWORTH, A., AND BROWN, R.—(1933) *J. Immunol.*, **24**, 349.
ZECKWER, I. T., AND GOODELL, H.—(1925) *J. Exp. Med.*, **42**, 43, 57.

THE TOXICITY FOR MICE OF CERTAIN FRACTIONS ISOLATED FROM *BACT. AERTRYCKE.*

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THERE is a voluminous literature dealing with attempts to isolate the so-called endotoxins of bacteria—those toxic constituents of bacterial cells that are not liberated freely into a fluid culture medium, and so cannot be separated in high concentration by simple filtration. But the methods hitherto employed have seldom been such as to allow of the isolation of the toxic material in a state approaching chemical purity. The present paper records an attempt

to isolate a toxic fraction from *Bact. aertrycke*, using mice as the test animals.

In reviewing briefly the observations of earlier workers we may omit reference to studies on bacteria other than those of the group to which *Bact. aertrycke* belongs and methods of extraction, such as simple autolysis, grinding with sodium chloride, etc., which yield only a crude bacterial extract.

Ecker (1917) reported that filtrates of 24-hour broth cultures of *Bact. paratyphosum B* were toxic for rabbits, and Ecker and Richardson (1925) obtained toxic filtrates from cultures of *Bact. suipestifer* in a synthetic medium. Ecker and Rimington (1928) extended this work along chemical lines. They grew *Bact. aertrycke* for 3–5 days in a medium consisting of 0·6 per cent. ammonium tartrate, 0·2 per cent. di-potassium hydrogen phosphate and 0·5 per cent. sodium chloride at pH 7·0–7·2. Filtrates of these cultures through Berkefeld candles when injected intravenously into rabbits in doses of 2–3 c.c. produced panting, paralysis of the hind legs and sometimes death in about 1½ hours. Addition of aluminium hydroxide, colloidal iron or kaolin, or the precipitation of the phosphates in the medium did not remove the toxicity. The toxin could be removed by adsorption on charcoal, but could not be recovered by elution. An attempt was made to concentrate the toxin by precipitation with neutral lead acetate, decomposition of the lead precipitate with ammonium sulphate and removal of the ammonium salts by dialysis. The solution so obtained was toxic for rabbits, but apparently not markedly so, since the protocols refer to two rabbits both of which recovered. Analysis of the final preparations showed small amounts of nitrogen and gave many of the usual tests for carbohydrates. The records show that the original cultures could be heated at 100° C. for 10 minutes without destroying the toxicity of the filtrates.

Branham (1925) confirmed the toxicity of filtrates of broth cultures of Salmonella organisms for rabbits and mice and the thermostability of the toxic substance.

Menten and King (1930) obtained a precipitate from a filtrate of a five-day broth culture of *Bact. aertrycke* by the addition of 2·5 per cent. acetic acid. This precipitate was purified by repeated solution in alkali and re-precipitation with acid. Further precipitation from a neutral solution by the addition of two volumes of 95 per cent. alcohol followed by solution in alkali and re-precipitation with acid gave a white substance, 0·03–0·05 mgm. of which produced acute illness in a rabbit but did not kill it. This illness was associated with a rise in blood-sugar—a question that has been dealt with more fully in the preceding paper. The toxic fraction isolated in this way did not reduce Benedict's solution, but did so after hydrolysis. It contained 11–12 per cent. of nitrogen, but gave negative tests for phosphorus and sulphur.

Casper (1929) isolated a toxic fraction from *Bact. aertrycke* by solution of the bacterial bodies in antiformin followed by precipitation with two volumes of alcohol. This fraction was further purified by repeated alcoholic precipitation from acid and alkaline solution. The substance so isolated was found to be toxic for mice; 1 c.c. of solution (the weight of substance is not given) killed mice regularly in 5–15 minutes with anaphylactic symptoms, 0·5 c.c. killed about half the mice injected in 5–20 minutes, the remainder recovered

in 30–60 minutes. It may be noted that this acutely toxic effect bears no resemblance to the results produced by the inoculation of the toxic fractions with which this report is concerned. The substance is recorded as giving a very weak biuret reaction, a doubtful reaction with picric acid, and a variable reaction with sulphosalicylic acid.

The findings of Boivin and his colleagues (1933, 1934) have been referred to in the preceding report on the isolation of immunizing fractions from *Bact. aertrycke*. Although the details of their toxicity tests are very scanty, it seems most probable that their toxic product is identical with, or closely related to, that isolated during the present investigation.

EXPERIMENTAL.

The object of this investigation was to isolate, in a state as nearly approaching chemical purity as possible, the substance or substances responsible for the toxic action of *Bact. aertrycke* on mice. In selecting a starting material it was clearly desirable to study the distribution of the toxic substance in the cells of a bacterial culture and the filtrate derived from it; since, though bacilli of this group are commonly regarded as endotoxin producers, certain of the observations recorded above indicate that the toxin may be set free to a considerable extent in the fluid of the culture medium, at least when incubation is continued sufficiently long for some autolysis to occur.

Preliminary tests in which filtrates from cultures in broth or in a synthetic medium were compared with suspensions of killed bacterial cells showed quite clearly that the greater part of the toxic material remained in the bodies of the bacterium and was not set free in the culture medium. If any considerable amount of this substance was to be separated for more detailed study, it seemed clear that the bacterial cells rather than culture filtrates should be employed. Since it was desired to avoid the use of such violent chemical reagents as antiformin, the cells were brought into solution, after killing and extracting with acetone, by digestion with a proteolytic enzyme solution obtained by filtering broth cultures of a spore-bearing bacillus, or, later, by digestion with trypsin. The lysate prepared by either method was found to be actively toxic.

Table I shows the deaths among groups of ten mice injected with falling

TABLE I.—*Showing the Numbers of Deaths among Groups, each of* 10 *Mice, Injected with Falling Doses of Bacterial Bodies and of Digests prepared from them.*

Dose (mgm.).	Undigested bacilli.	Bacterial enzyme digest.	Tryptic digest.
2·0	10	10	10
1·0	10	8	10
0·5	5	3	3
0·25	6	1	5
0·125	3	0	3

doses of acetone extracted bodies, a digest prepared with the bacterial enzyme and a digest prepared with trypsin. The doses are given in terms of the weight of substance injected. Injections were given intraperitoneally. The mice were observed for seventy-two hours, since few deaths were found to occur after this period.

The digests so obtained were treated in various ways in an attempt to isolate the toxic substance in a purer form.

Experiments in which the digests were treated with colloidal iron gave somewhat variable results. In most instances the toxicity was little, if at all, reduced, but in some the digest so treated, and the precipitates obtained from it, were definitely less toxic than untreated preparations, and this step was therefore abandoned.

The toxic material could be precipitated from the digest by precipitation with alcohol (6 volumes) or acetone (6 volumes). The alcohol-soluble fraction was tested and found to be non-toxic in a dose of 10 mgm. The alcohol precipitate was dissolved in water and treated with basic lead acetate. The supernatant fluid after removal of the precipitate showed a considerable decrease in toxicity, but the toxin could not be recovered from the lead precipitate by treatment with H_2S.

At this stage it had become evident that the toxic substance was contained in the same fractions as those that were found by Prof. Raistrick and Prof. Topley to induce an active antibacterial immunity. Ten grammes of acetone-treated bacteria were therefore fractionated and refractionated in the manner described in their report, and the fractions tested by injecting groups of 10 mice with falling doses. The results are set out in Table II. Fractions F68, F68/U, etc., are named as in the two preceding papers. F95 is the fraction precipitated by increasing the alcohol concentration from 68 to 95 per cent. after separating the original 68 per cent. precipitate from the tryptic digest. All injections were given intraperitoneally.

TABLE II.—*Showing the Numbers of Deaths in Groups of 10 Mice after Injection with Falling Doses of Various Fractions of Bact. aertrycke.*

Dose (mgm.).	Fraction tested.						
	Bacterial bodies.	F68.	F68/U.	F68/50.	F68/60.	F68/68.	F68/95.	F95.
8	9	10	..	..	..	..	10	0
2	10	10	10	10	10	10	1	0
0·5	10	5	5	10	7	9	1	0
0·125	1	4	0	2	1	0	0	0
0·03125	0	0	0	0	0	0	0	0

It will be noted that all the fractions F68/U, F68/50, F68/60 and F68/68, prepared by fractionating the original 68 per cent. alcohol precipitate from the cleared tryptic digest, showed approximately the same degree of toxicity as that precipitate itself (F68) and as the standard acetone-treated bacteria. There is a suggestion that F68/U is slightly less toxic than the others, but such

a difference in mortality is insignificant in groups of 10 mice. The average lethal dose of each of these fractions would appear to fall within the range 0·125–0·5 mgm.—probably rather nearer the larger than the smaller figure.

The precipitate obtained from the tryptic digest by increasing the alcohol concentration from 68 per cent. to 95 per cent. (F95) is entirely non-toxic. It has been found, as reported in the preceding paper by Raistrick and Topley, to be non-immunizing. Fraction F68/95 is slightly toxic, but much less so than the fractions F68/U–F68/68. Its immunizing potency was found to be insignificant.

It will be noted that there is no evidence of any concentration of the toxic material in the partially purified fractions; their average lethal dose is no smaller than that of the acetone-treated bacteria from which they were derived, This is not surprising, even on the view that the total toxic effect of the bacterial cells is due to the toxin present in the fractions. The original 68 per cent. alcohol precipitate obtained from 10 gm. of bacterial bodies weighed 2·3 gm.; and of 2 gm. of this material, 1·01 gm. fell in the toxic fractions F68/U, F68/50, F68/60 and F68/68 on refractionation with alcohol. The concentration by weight was, therefore, at most eight to nine-fold, and a variation within this range might not be detected in tests on groups of 10 mice. It is, moreover, a large assumption that the toxicity of the substance remains entirely unaltered during the treatment it receives. The evidence is, therefore, compatible with the view that the toxic substance in our fractions represents the total toxin content of the bacterial cells, but it is at least possible that additonal toxic substances are present that have escaped detection in the method of fractionation we have employed. If this is so it seems likely that they are of minor importance, since the toxic effect of the whole bacterial cells is reproduced so closely by the fractions we have isolated from them.

As regards the way in which the toxin acts we have as yet little information. After injection of relatively large doses (1–2 mgm.) of the acetone-treated bacilli or of any one of the toxic fractions, the first deaths occur after 5–6 hours, and the majority of deaths within 24 hours. At necropsy no striking naked-eye changes are found beyond some injection of the smaller blood-vessels in the peritoneal cavity and, in some but not in all cases, a slight reddening of the suprarenal glands. Sections from these glands, even when no changes have been detected with the naked eye, have shown some dilatation of the medullary vessels, a rather striking increase in the numbers of large mononuclear cells and sometimes of the polymorphonuclear leucocytes, and early degenerative changes in the medullary cells themselves. These changes have not, however, been sufficiently marked to justify any definite conclusions in the absence of the examination of further material by more specialized histological methods.

It is probable that the mechanism of toxæmia in the mouse is similar, in its general character, to that induced in the rabbit. Prof. Topley, in unpublished experiments, has found that sub-lethal doses of insulin greatly increase the rapidity with which mice die after the injection of a moderate dose of killed *Bact. aertrycke*, and, to a less extent, increase the numbers of deaths; and Prof. Delafield has found in some instances a marked decrease in the blood-sugar of mice injected with killed *Bact. aertrycke* and maintained thereafter at

a temperature of 35° C. This would seem to correspond to the secondary hypoglycæmic phase that occurs in rabbits suffering from the severer types of toxæmia. Whether a primary hyperglycæmia occurs in the mouse has not yet been determined.

The chemical characters of the various toxic fractions have been described in the preceding paper by Raistrick and Topley.

REFERENCES.

Boivin, A., and Mesrobeanu, I. and L.—(1933) *C. R. Soc. Biol.*, Paris, **114**, 307.—(1934) *Ibid.*, **115**, 304.
Branham, S. E.—(1925) *J. Infect. Dis.*, **37**, 291.
Casper, W.—(1929) *Z. Hyg. InfektKr.*, **109**, 170.
Ecker, E. E.—(1917) *J. Infect. Dis.*, **21**, 541.
Idem and Richardson, M. L.—(1925) *Ibid.*, **37**, 538.
Idem and Rimington, C.—(1928) *J. Hyg.*, Camb., **27**, 44.
Menten, M. L., and King, C. G.—(1930) *J. Infect. Dis.*, **46**, 275.